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HER-2 growth factor receptor pathways are strongly implicated in the clinical progression of breast cancer. We have targeted these receptors for therapeutic intervention, using humanized monoclonal antibody to HER-2 receptor (Herceptin). To assess how best to use Herceptin alone and in combination with chemotherapeutic agents, studies were designed to evaluate its inhibitory effects on cancer growth. The nature of interactions between Herceptin and cytotoxic drugs was evaluated by multiple drug analysis, with *in vitro* results showing that Herceptin has synergy with cisplatin and thiotepa and additive cytotoxicity with doxorubicin and paclitaxel. Herceptin combined with paclitaxel, doxorubicin or cyclophosphamide *in vivo* resulted in significant reductions in breast cancer volumes as compared to chemotherapy alone controls. The additive or synergistic interaction of Herceptin with alkylating agents, cisplatin, taxanes, and anthracyclines in HER-2-expressing breast cancer cells suggests that these are rational combinations to take to human trials. Preclinical studies also suggest that Herceptin, by targeting an alternative growth or survival pathway, may provide a new therapeutic option for some patients for whom antiestrogen therapy is not effective. Evaluation of biologic mechanisms underlying cisplatin-antibody synergy suggest that p21, a critical modulator of cell cycle arrest prior to the onset of DNA repair, is altered by Herceptin treatment in breast cancer cells with HER-2-overexpression.

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Richard J. Dutton 11/20/98

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(2) Pietras, R.J. (1997). HER-2 tyrosine kinase pathway regulates estrogen receptor and growth in human breast cancer cells. Proceedings DOD Breast Cancer Research Program 3: 987-988.

(3) Pietras, R.J., M. Pegram, R. Finn, D. Maneval and D. Slamon (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive agents. Oncogene 17: 2235-2249

(4) Pegram, MD, S. Hsu, R. Pietras, M. Sliwkowski, M. Beryt, D. Coombs, D. Baly, F. Kabbinavar and D. Slamon (1998). Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene (in press).

(5) Pietras, R.J. and M. Pegram (1998). Oncogene activation and breast cancer progression. Contemporary Endocrinology, 11 (in press).

(6) Aguilar, Z., R. Finn, M. Pegram, B. Ramos, F. Kabbinavar, R.J. Pietras, R. Akita, M. Sliwkowski and D. Slamon (1999). Biologic effects of heregulins on normal and malignant human breast and ovarian epithelial cells. (To be submitted; in preparation).

(7) Pietras, R.J., P.N. Wongvipat and D.J. Slamon (1999). Reversal of tamoxifen resistance in HER-2/neu-overexpressing human breast cancer cells using HER-2/neu antibody. (To be submitted; in preparation).

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INTRODUCTION

Herceptin®, the first successful cancer treatment that targets a specific gene alteration in breast cancer cells, was approved by the FDA on September 25, 1998. This drug is a humanized monoclonal antibody directed against the external domain of HER-2 receptor (rhuMAb HER-2), a protein overexpressed in 25-30% of human breast cancers and associated with poor clinical outcome (20,22,23,25,28). The drug bears the generic name, trastuzumab, and is indicated for use in patients with metastatic breast cancer as a first-line therapy in combination with chemotherapy and also as a single agent. This FDA approval validates the concept that growth factor receptors that regulate breast cancer cell growth can be an important target for new cancer treatments (16). Experiments funded by this grant lead to the discovery that activation of growth factor receptors by Herceptin® enhances the sensitivity of cells to drugs that damage DNA and, thereby, potentiates tumor cell death (20,24,26).

Approximately 30% of human breast cancers have amplification and/or overexpression of HER-2 gene which encodes a cell surface growth factor receptor. We have confirmed earlier observations showing that monoclonal antibodies to HER-2 receptor have a cytostatic effect in suppressing growth of breast cancer cells with overexpression of HER-2 gene product. In order to elicit a cytocidal effect, therapy with anti-receptor antibody was used in combination with the DNA-damaging drug, cisplatin, and this combined treatment produced a synergistic decrease in cell growth which was significantly different from the effects of either antibody or cisplatin given alone (1,21,24). Moreover, on testing the use of repeated, cyclic doses of cisplatin in combination with rhuMAb HER-2, we found a more profound anticancer effect (24,26). We found that the order of antibody-drug administration was critical and clearly affected the magnitude of observed antitumor responses in HER-2-overexpressing human breast cancer xenografts (24,26). The schedule and timing of therapeutic agents proved important in achieving synergistic killing of tumor cells in the clinic (3,24,26).

Recent clinical findings suggest that overexpression of HER-2 oncogene may be involved in determining the sensitivity of human cancers to chemotherapeutic agents (5,17,23). To define the effect of HER-2 oncogene expression on sensitivity to chemotherapeutic drugs, *in vitro* dose-response curves following exposure to 7 different classes of chemotherapeutic agents were compared for HER-2 and control-transfacted cells (17). Chemosensitivity was also tested *in vivo* for HER-2 and control-transfected human breast cancer xenografts in athymic mice. These studies indicate that HER-2 overexpression alone was not sufficient to induce intrinsic, pleomorphic drug resistance. In addition, changes in chemosensitivity profiles resulting from HER-2 overexpression were cell line-specific in vitro. Under *in vivo* treatment conditions, HER-2-overexpressing breast cancer xenografts were responsive to different classes of chemotherapeutic drugs as compared to control xenografts (17). We found no statistically significant differences in chemosensitivity between HER-2-overexpressing and control tumors. However, HER-2-overexpressing tumor xenografts exhibited more rapid regrowth than control xenografts following the initial response to chemotherapy, suggesting that a high rate of tumor cell proliferation rather than intrinsic drug resistance may be responsible for the adverse prognosis associated with HER-2 overexpression in human breast cancers. It appears that the growth stimulus afforded by overexpression of HER-2 receptor allows for the rapid proliferation of any surviving cells following treatment with chemotherapy. This may, in turn, allow the emergence of acquired chemotherapeutic drug resistance through the processes of clonal or adaptive selection of resistant tumor cells. If, as our experimental data suggest, the adverse prognosis seen in patients with tumors with HER-2-overexpression is due to rapid tumor cell proliferation rather than *de novo* resistance to chemotherapy, then maximizing the reduction in tumor burden with more active chemotherapeutic agents and/or higher dose intensity may result in improved clinical responses. This hypothesis is consistent with recent findings in clinical trials (17) and suggests that assay of HER-2 levels in malignant breast tissue is important in the selection of effective treatment regimens for affected patients.

Members of both steroid and peptide receptor classes are important prognostic factors in human breast cancer (5,22). Clinical data indicate that overexpression of the HER-2 gene is associated with an estrogen receptor-negative phenotype. We have demonstrated that introduction of a HER-2 cDNA, converting non-overexpressing breast cancer cells to those which overexpress this receptor, results in development of estrogen-independent growth that is insensitive to both estrogen and the antiestrogen, tamoxifen. Moreover, activation of HER-2 receptor in breast cancer cells by the peptide growth factor, heregulin (4,12), leads to direct and rapid phosphorylation of ER on tyrosine residues. This is followed by interactions between ER and estrogen-response elements in the nucleus and production of an estrogen-

induced protein, progesterone receptor (PR). With long-term exposure to HRG, down-regulation of ER and, in turn, PR occurs, producing an ER-/PR- phenotype (22). These data demonstrate a direct link between these two receptor pathways and suggest one mechanism for genesis of endocrine resistance in breast cancers. Since overexpression of HER-2 receptor in breast cancer predicts a poor response to endocrine therapy, understanding the relationship between HER-2 and ER receptors may facilitate patient management and the development of more effective therapies.

EXPERIMENTAL RESULTS

This report presents data for the reporting period from 1 November 1997 to 30 October 1998. The experimental results will be presented here with reference to the goals outlined in the original Statement of Work. We have made further progress in studies of the therapeutic advantage of treatment with humanized monoclonal antibody to HER-2 receptor (rhuMAb HER-2 /or Herceptin®) in combination with chemo-therapeutic drugs. As noted above, this work has contributed, in part, to FDA approval of Herceptin® for use in the treatment of patients with metastatic breast cancer.

Task 1) Dose and schedule of humanized monoclonal antibody to HER-2 receptor on growth of human breast cells : Task completed. No additional work from 1 November 1997 to 30 October 1998.

Task 2) Conditions for maximal tumor cell killing by cisplatin in combination with monoclonal antibody to HER-2 : Task completed. No additional work from 1 November 1997 to 30 October 1998.

Task 3) Sensitivity of cells with overexpression of HER-2 to chemotherapeutic drugs (including drug/antibody synergy and molecular mechanisms):

Chemotherapeutic drug interactions with Herceptin®

To determine how best to use Herceptin® both alone and in combination with other therapeutic agents, we undertook a series of studies to evaluate its inhibitory effects in both *in vitro* and *in vivo* preclinical models. As noted above, our investigations showed that use of cisplatin in combination with Herceptin® potentiated cytotoxicity of the chemotherapeutic agent by decreasing DNA repair activity following cisplatin-induced DNA damage (21,24). This effect, termed receptor-enhanced chemosensitivity, specifically targets HER-2-overexpressing cells and has been shown to be synergistic, resulting in a two-log increase in cisplatin-induced cytotoxicity as well as full pathologic remission in experimental animals bearing HER-2-overexpressing human breast cancer xenografts (21,24). To further characterize the nature of the interaction between Herceptin® and other classes of cytotoxic drugs, we used the multiple drug analysis method to determine combination index (CI) values for a variety of chemotherapeutic agent / Herceptin® combinations *in vitro* (13,17,19). SKBR3 human breast cancer cells with HER-2 amplification served as the target cell line for *in vitro* cytotoxicity experiments:

Synergistic interactions observed for Herceptin® in combination with:

cisplatin (CI=0.48)
thiotepa (CI=0.67)
etoposide (CI=0.54)

Additive cytotoxic effects observed with Herceptin® in combination with:

doxorubicin (CI=1.16)
paclitaxel (CI=0.91)
methotrexate (CI=1.15)
vinblastine (CI=1.09)

5-fluorouracil (CI=2.87)

In vivo therapeutic studies were conducted with HER-2-overexpressing MCF-7 human breast cancer cells which, in contrast to SKBR3 cells, are tumorigenic in athymic mice. Combinations of Herceptin® with doxorubicin, paclitaxel, methotrexate, etoposide and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone controls ($P<0.05$). Xenografts treated with Herceptin® plus 5-fluorouracil were not significantly different from 5-fluorouracil alone controls consistent with the subadditive effects observed in the *in vitro* studies. The additive or synergistic therapeuitc interaction of rhuMAb HER-2 with alkylating agents, platinum analogues, taxanes, anthracyclines, topoisomerase II inhibitors and some antimetabolites in HER-2-overexpressing human breast cancer cells suggests that these are rational combinations to take to human clinical trials (19).

Biologic basis of interactions between chemotherapeutic drugs and Herceptin®

To further explore the molecular basis for these observations of Herceptin®-drug interactions leading to increments in cell sensitivity to DNA-damaging drugs after antireceptor antibody treatment (1,7,11,21), we investigated potential pathways leading to suppression of DNA repair are outlined in Figure 1.

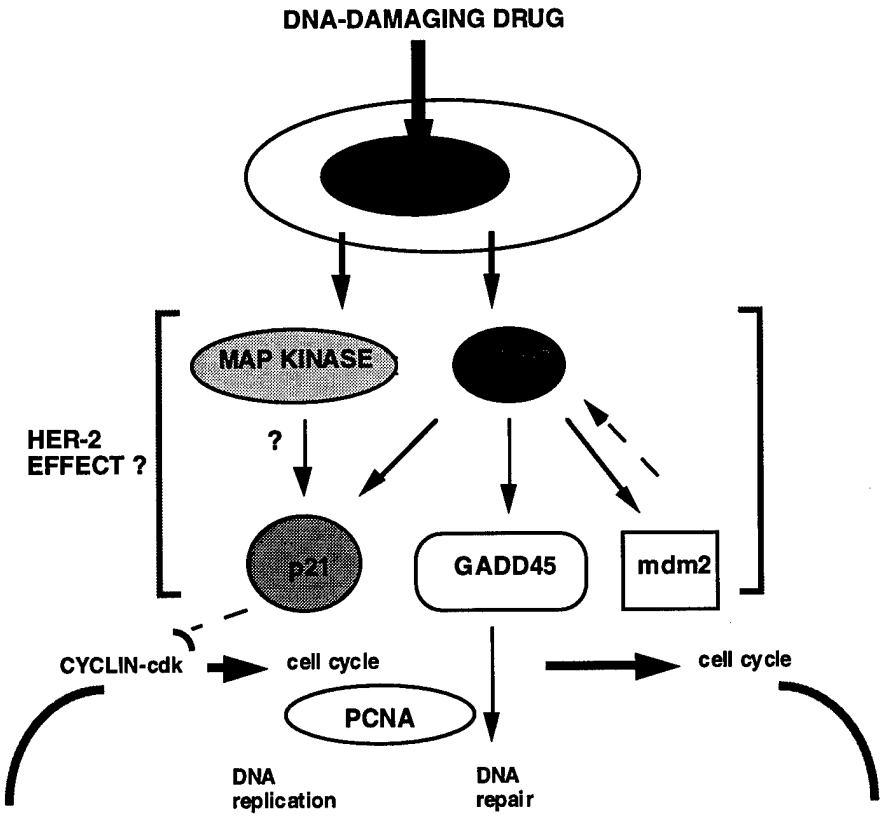

FIG. 1. Potential pathways for HER-2 receptor-mediated blockade of DNA repair. Treatment of breast cells with a DNA-damaging drug normally activates p53 and p21, leading to cell cycle arrest and initiation of DNA repair for preservation of DNA integrity (6,8,14,15,29). However, pre-treatment of cells with antibody to HER-2 receptor elicits blockade of DNA repair after exposure to DNA-damaging drugs, leading to reduced DNA integrity and greater cell death. The pathway triggered by antireceptor antibody may interfere with p21 activity (6,10) or that of other signal molecules involved in regulation of DNA repair (see MAP kinase, GADD 45, mdm2, PCNA in scheme above).

The tumor suppressor gene p53 is known to be a critical mediator of the cellular response to DNA damage (6,10,26,28-30). Induction of the CDK inhibitor, p21WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism that requires interaction of the p53 protein with a p53-binding site in the p21WAF1 promoter (6,10). Recent studies, however, have shown that induction of p21WAF1 following growth factor stimulation may not always require p53 and may instead be directly activated by mitogen-activated protein kinase (14). Consistent with this is the observation that withdrawal of growth factors *in vitro* is associated with down-regulation of p21WAF1 expression and with enhanced cell killing in response to DNA damage (6). It is known that basal levels of p21WAF1 are not sufficient to cause cell cycle arrest and that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest. This results in a reduced time for completion of DNA repair. To assess the activity of p21WAF1 in response to DNA damage in MCF-7/HER-2 cells in the presence or absence of the anti-HER-2 antibody, we first performed Northern blot analyses of p21WAFI expression levels. MCF-7/HER-2 cells were treated with rhuMAb HER-2 alone or prior to cisplatin exposure. Parallel cells were treated with either control solution alone or cisplatin alone. At 6h, 12h and 24h, cells were processed for RNA extraction and determination of p21WAF1 transcript levels. As expected, progressive induction of p21WAF1 transcripts was seen at 6-24h post-cisplatin treatment. However, increased levels of p21WAF1 transcript were not sustained in MCF-7 HER-2 cells which had been exposed to cisplatin in the presence of rhuMAb HER-2 (26). Although p21WAF1 transcript level increases at 6-12h, it is comparable to baseline levels by 24 h. Moreover, the level of p21WAF1 at 24 h is markedly less than the levels seen after cisplatin given without antibody (see FIG. 2). A clear reduction in the basal level of p21WAF1 also occurred after 12-24 h exposure to antibody alone when compared to controls (FIG. 2). Another transcript, cyclin D1, showed no variation with antibody, cisplatin or combination therapy (26).

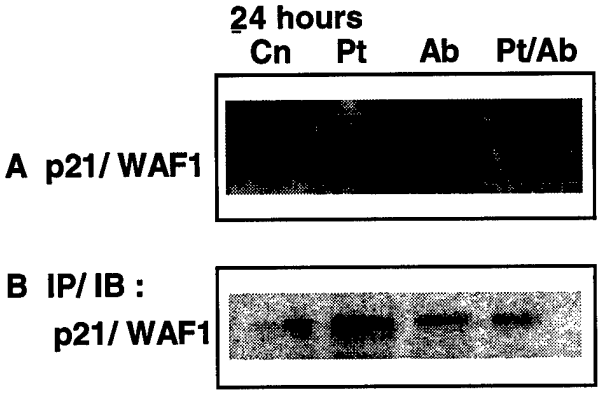

FIG. 2. Herceptin® (recombinant humanized monoclonal antibody to HER-2 receptor / rhuMAbHER-2) alters p21WAF1 transcript and protein levels after cisplatin treatment of human breast cancer cells with HER-2 overexpression. (A) MCF-7 HER-2 cells were treated with control solution (Cn), cisplatin alone (Pt), 200 µg/ml rhuMAb HER-2 alone (Ab), or 200 µg/ml rhuMAb HER-2 in combination with cisplatin (Pt /Ab). After 24 hours, cells were processed for preparation of RNA and determination of p21WAF1 transcripts using Northern blot (probe for p21/WAF1 generously provided by Dr. Bert Vogelstein). (B) MCF-7 HER-2 cells were treated with control solution (Cn), cisplatin alone (Pt), 200 µg/ml rhuMAb HER-2 alone (Ab), or 200 µg/ml rhuMAb HER-2 in combination with cisplatin (Pt /Ab). After 24 hours, cells were processed for Western blot analysis and determination of p21WAF1 levels by immunoprecipitation with anti-p21/WAF1 antibody, followed by immunoblot with anti-p21/WAF1 antibody (26). See text for additional details.

Western analyses of the level of p21WAF1 protein in MCF-7/HER-2 cells likewise show enhanced amounts of the protein at 6-24 h after cisplatin (26), however, consistent with the Northern blot studies, treatment of cells with antireceptor antibody elicits a reduced level of p21WAF1 protein under basal conditions and blunts the anticipated response to cisplatin therapy at 12-24 h, as compared to controls. These results are consistent with independent reports on depletion of p21WAF1 after withdrawal of growth factors (6) and suggest an important role for growth factor pathways in modulating the activity of some proteins which regulate the cell cycle in response to DNA damage. In view of the crucial role of tyrosine

phosphorylation in regulating the activity of diverse signaling molecules (23,27-29), we also plan to assess the potential influence of the HER-2 receptor pathway on phosphorylation of tyrosine residues in p21WAF1 (26). Our prime objective in this work remains to firmly establish the contribution of DNA repair in receptor-modulated sensitivity of cancer cells to DNA-damaging drugs. This would provide a good biologic rationale for pursuit of combined drug-antibody therapy in the clinic (16,23,28). Our evaluation of molecular mechanisms involved in this phenomenon (FIG. 1) suggest that p21, a critical modulator of cell cycle arrest prior to the onset of DNA repair (6), is affected by Herceptin® treatment in MCF-7/HER-2 cells.

Task 4) Impact of HER-2 gene expression on cell sensitivity to estrogen and antiestrogens:

As reported previously, we have demonstrated that overexpression of HER-2 cDNA or stimulation of HER-2 receptor with heregulin ligand results in development of estrogen-independent growth of human breast cancer cells (20,22). These data indicated a link between two receptor pathways, ER and HER-2, and suggested one mechanism for development of endocrine resistance in human breast cancers. Nass et al. (16) suggest that such findings indicate that Herceptin® may "provide a new therapeutic option for some patients for whom antiestrogen therapy is not effective". To determine whether Herceptin® can influence the cell response to antiestrogen therapy with tamoxifen, we conducted a series of experiments with MCF-7 human breast cancer xenografts in nude mice (FIG. 3). MCF-7 cells with or without overexpression of HER-2/*neu* receptor were grown as subcutaneous xenografts as before (20,21). These *in vivo* studies demonstrate a clear growth-inhibitory therapeutic benefit of Herceptin® combined with tamoxifen in HER-2/*neu*-overexpressing human breast cancer xenografts (FIG. 3).

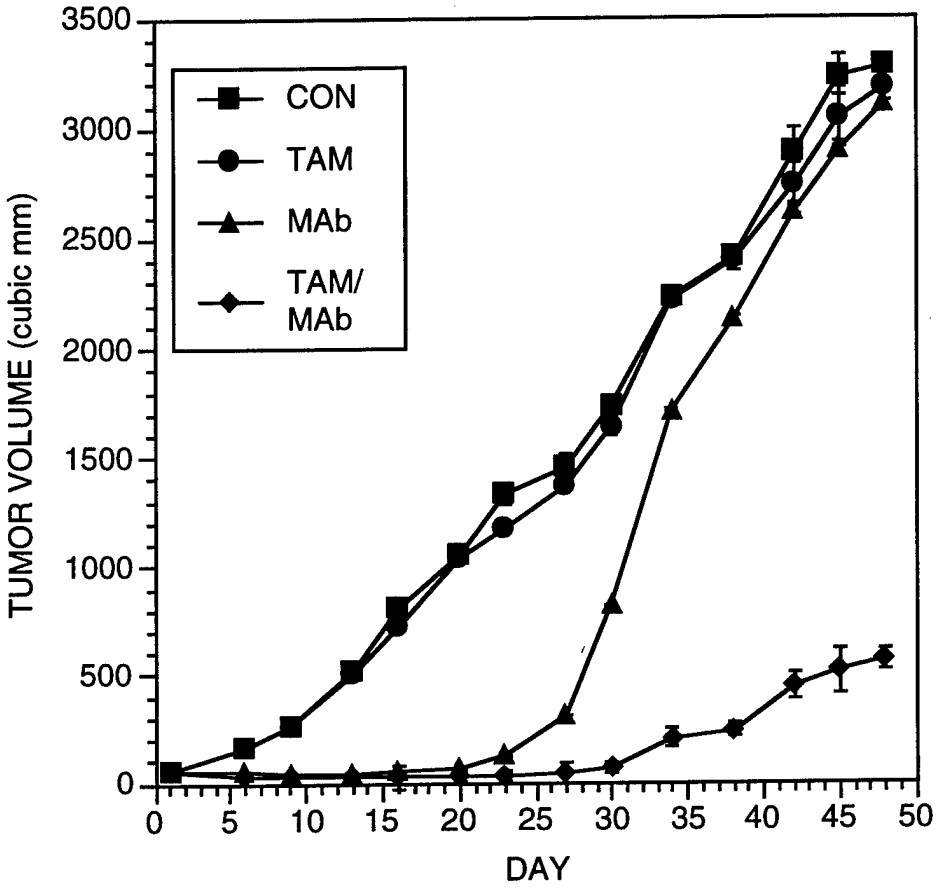

FIG.3. Reversal of tamoxifen resistance in human breast cancer cells with HER-2/*neu* overexpression. The therapeutic benefit of tamoxifen with Herceptin® was evaluated using MCF-7/HER-2 breast cancer

cells grown as xenografts. The resulting tumors were treated with control injection (CON), tamoxifen (TAM, 5 mg sustained release pellet sc), Herceptin®[monoclonal antibody to HER-2/*neu* receptor] (MAb) alone (10 mg/kg every 4 days) or Herceptin® in combination with tamoxifen (TAM/MAb).

To pursue this work further, we and collaborators at UCLA (2) have also used receptor-specific ELISA assays and quantitated the amount of each member of the class I receptor tyrosine kinase family (HER-1, HER-2, HER-3, HER-4) in six different human breast cancer cells (ER+ and ER-) with and without HER-2 overexpression. We find that HER-2 overexpression itself affected the expression of the other three class I receptors and that cells expressing the highest levels of HER-2 and HER-3 had the greatest response to heregulin. These results may promote further progress in understanding the interactions between class I tyrosine kinase receptors and estrogen receptor pathways in human breast cancer cells.

Task 5) Conditions for maximal breast tumor cell killing by alkylating agents (cyclophosphamide, thiotepa) in combination with humanized monoclonal antibody to HER-2 receptor in vitro and in vivo:

As noted above, to characterize the nature of the interaction between Herceptin® and other classes of cytotoxic drugs, we used the multiple drug analysis method to determine combination index (CI) values for a variety of chemotherapeutic agent / Herceptin® combinations *in vitro* (19,21). SKBR3 human breast cancer cells with HER-2 amplification served as the target cell line for *in vitro* cytotoxicity experiments, and clear synergistic interactions were observed for the combination of Herceptin® with thiotepa (CI=0.67). *In vivo* therapeutic studies were conducted with HER-2-overexpressing MCF-7 human breast cancer cells which are tumorigenic in athymic mice (19). Combinations of Herceptin® with cyclophosphamide *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone controls ($P<0.05$). The synergistic therapeutic interaction of rhuMAb HER-2 with alkylating agents in HER-2-overexpressing human breast cancer cells suggests that these are rational combinations to take to human clinical trials.

CONCLUSIONS

In summary, substantial progress has been made in studies of the therapeutic advantage of treatment with Herceptin® and chemotherapeutic agents or antihormone drugs. Since HER-2 receptor pathways are strongly implicated in the clinical progression of breast cancer, we have targeted these receptors for therapeutic intervention, using humanized monoclonal antibody to HER-2 receptor (Herceptin). To assess how best to use Herceptin alone and in combination with chemotherapeutic agents, studies were designed to evaluate its inhibitory effects on cancer growth. Interactions between Herceptin and cytotoxic drugs as revealed from multiple drug analysis indicate that Herceptin has synergy with cisplatin and thiotepa and additive cytotoxicity with doxorubicin and paclitaxel. Herceptin combined with paclitaxel, doxorubicin or cyclophosphamide *in vivo* resulted in significant reductions in breast cancer volumes as compared to chemotherapy alone controls. The additive or synergistic interaction of Herceptin with alkylating agents, cisplatin, taxanes, and anthracyclines in HER-2-expressing breast cancer cells suggests that these are rational combinations to take to future human trials. This preclinical work has contributed, in part, to the recent FDA approval of Herceptin for treatment of women with metastatic breast cancer (16,18,28).

Preclinical studies from this award also suggest that Herceptin, by targeting an alternative growth or survival pathway, may provide a new therapeutic option for some patients for whom antiestrogen therapy is not effective. In addition, evaluation of biologic mechanisms underlying cisplatin-antibody synergy suggest that p21, a critical modulator of cell cycle arrest prior to the onset of DNA repair, is altered by Herceptin treatment in breast cancer cells with HER-2 overexpression. Despite the termination of this award, we plan to continue our laboratory studies as required to promote further progress in this clinical effort at UCLA and other clinical research centers. We hope that work on the elucidation of the molecular mechanism underlying the synergistic effect of Herceptin and DNA-reactive drugs (25,26) will promote further progress in this new therapeutic initiative. We thank you for your support of our research.

REFERENCES

1) Aboud-Pirak E., E. Hurwitz, M. Pirak, F. Bellot, J. Schlessinger and M. Sela (1988). Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in nude mice. J. Natl. Cancer Inst., 80:1605.
2) Aguilar, Z., R. Finn, M. Pegram, B. Ramos, F. Kabbinavar, R.J. Pietras, R. Akita, M. Sliwkowski and D. Slamon (1998). Biologic effects of heregulins on normal and malignant human breast and ovarian epithelial cells. (in preparation).
3) Arbuck S.G. (1994). Paclitaxel: What schedule? What dose? J. Clin. Oncol., 12 : 233.
4) Bacus S.S., E. Huberman, D. Chin, K. Kiguchi, S. Simpson, M. Lippman and R. Lupu (1992). A ligand for the erb B-2 oncogene product (gp30) induces differentiation of human breast cancer cells. Cell Growth & Differentiation, 3 : 401.
5) Benz C., G. Scott, J. Sarup, R. Johnson, D. Tripathy, E. Coronado, H. Shepard & C. Osborne (1993). Estrogen-dependent, tamoxifen-resistent tumori-genic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res.Treatment, 24 : 85.
6) Canman, C.E., Gilmer, T.M., Coutts, S. & Kastan, M. (1995). Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes & Development, 9 : 600-611.
7) Christen R., D. Hom, A. Eastman and S. Howell (1991). Epidermal growth factor regulates ability of human ovarian carcinoma cells to repair DNA damage. Proc. AACR, 32 : 430.
8) Chu G. (1994). Cellular responses to cisplatin. J. Biol. Chem., 269 : 787.
9) Dominici C., A. Alimonti, S. Caroli et al.(1986).Chemotherapeutic agent cisplatin monitoring in biological fluids by inductively-coupled plasma emission spectrometry. Clin. Chim. Acta, 158 : 207.
10) El-Deiry W., T. Tokino, V. Velculescu, D. Levy, R. Parsons, J. Trent, D. Lin, W. Mercer, K. Kinzler and B. Vogelstein (1993). WAF1, a potential mediator of p53 tumor suppression. Cell, 75: 817.
11) Hancock M.C., B.C. Langton, T. Chan et al. (1991). A monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of cisdiamminedichloroplatinum against human breast and ovarian tumor cell lines. Cancer Res., 51 : 4575.
12) Holmes W.E., M.X. Sliwkowski, R.W. Akita, W.J. Henzel, J. Lee, J.W. Park, D. Yansura, N. Abadi, H. Raab, G.D. Lewis, H.M. Shepard, W.-J. Kuang, W.I. Wood, D.V. Goeddel and R.L. Vandlen (1992). Identification of heregulin, a specific activator of p185erb B2. Science, 256 : 1205.
13) Hsu, S., M. Pegram, R. Pietras, M. Beryt and D. Slamon (1997). Therapeutic advantage of chemotherapy drugs in combination with recombinant, humanized anti-HER-2/neu monoclonal antibody (rhuMAb HER-2) against human breast cancer cells and xenografts with HER-2/neu overexpression. Proceedings AACR Conference on Basic and Clinical Aspects of Breast Cancer 3/12: A39.
14) Liu, Y., Martindale, J., Gorospe, M., and Holbrook, N. (1996). Regulation of p21WAF1/CIP1 expression through mitogen-activated protein kinase signaling pathway. Cancer Res., 56 : 31-35.
15) Montine T.J. and R.F. Borch (1988). Quiescent LLC-PK cells as a model for cis-diammine-dichloroplatinum nephrotoxicity and modulation by thiol rescue agents. Cancer Res., 48 : 6017.
16) Nass S., H. Hahm and N. Davidson (1998). Breast cancer biology blossoms in the clinic. Nature Medicine 4: 761-762.
17) Pegram, M.D., R. Finn, K. Arzoo, M. Beryt, R. J. Pietras and D. J. Slamon (1997). The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene 15: 537-547.
18) Pegram, M., A. Lipton, R. Pietras, D. Hayes, B. Weber, J. Baselga, D. Tripathy, T. Twadell, J. Glaspy and D. Slamon (1995). Phase II study of intravenous recombinant humanized anti-p185 HER-2 monoclonal antibody (rhuMAb HER-2) plus cisplatin in patients with HER-2/neu overexpressing metastatic breast cancer. Proc.Am.Soc.Clin.Oncol. 14 : 106.
19) Pegram, MD, S. Hsu, R. Pietras, M. Sliwkowski, M. Beryt, D. Coombs, D. Baly, F. Kabbinavar and D. Slamon (1998). Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene (in press).
20) Pietras, R.J. (1997). HER-2 tyrosine kinase pathway regulates estrogen receptor and growth in human breast cancer cells. Proceedings DOD Breast Cancer Research Program 3: 987-988.
21) Pietras, R.J., B.M. Fendly, V. Chazin, M.D. Pegram, S.B. Howell and D.J. Slamon (1994). Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene, 9 : 1829-1838.

22) Pietras, R.J., J. Arboleda, D. Reese, N. Wongvipat, M. Pegram, L. Ramos, C. Gorman, M. Parker, M.X. Sliwkowski, and D.J. Slamon (1995). HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, 10 : 2435-2446.
23) Pietras, R.J. and M. Pegram (1998). Oncogene activation and breast cancer progression. Contemporary Endocrinology 11 (in press).
24) Pietras, R.J., M. Pegram, R. Finn, D. Maneval and D. Slamon (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive agents. Oncogene (in press).
25) Pietras, R.J., P.N. Wongvipat and D.J. Slamon (1999). Antibody to HER-2/neu growth factor receptor reverses antiestrogen resistance in human breast cancer cells with overexpression of HER-2/neu oncogene. (in preparation).
26) Pietras, R.J., P.N. Wongvipat, H.J. Lee and D.J. Slamon (1999). Monoclonal antibody to HER-2 receptor modulates WAF1/p21 activity and the repair of cisplatin-induced DNA damage in human breast cancer cells. (in preparation).
27) Sarup J.C., R.M. Johnson, K.L. King, B.M. Fendly, M. T. Lipari, M.A. Napier, A. Ullrich and H. M. Shepard (1991). Characterization of an anti-p185HER2 monoclonal antibody that stimulates receptor function and inhibits tumor cell growth. Growth Regulation, 1 : 72.
28) Shepard H.M., G. Lewis, J. Sarup, B. Fendly, D. Maneval, J. Mordenti, I. Figari, C. Kotts, M. Palladino, A. Ullrich & D. Slamon (1991). Monoclonal antibody therapy of human cancer:Taking the HER2 oncogene to the clinic. J. Clin. Immunol., 11 : 117.
29) Tsai, C.-M., Levitzki, A., Wu, L.-H., Chang, K.-T., Cheng, C.-C., Gazit, A. & R.-P. Perng (1996). Enhancement of chemosensitivity by tyrphostin AG825 in high-p185neu expressing non-small cell lung carcinoma cells. Cancer Res., 56: 1068-1074.
30) Whitaker S.J. (1992). DNA damage by drugs and radiation. Eur. J. Cancer, 28 : 273.

APPENDIX

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The effect of HER-2/*neu* overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells

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Recent studies indicate that oncogenes may be involved in determining the sensitivity of human cancers to chemotherapeutic agents. To define the effect of HER-2/*neu* oncogene overexpression on sensitivity to chemotherapeutic drugs, a full-length, human HER-2/*neu* cDNA was introduced into human breast and ovarian cancer cells. *In vitro* dose-response curves following exposure to 7 different classes of chemotherapeutic agents were compared for HER-2- and control-transfected cells. Chemosensitivity was also tested *in vivo* for HER-2- and control-transfected human breast and ovarian cancer xenografts in athymic mice. These studies indicate that HER-2/*neu* overexpression was not sufficient to induce intrinsic, pleomorphic drug resistance. Furthermore, changes in chemosensitivity profiles resulting from HER-2/*neu* transfection observed *in vitro* were cell line specific. *In vivo*, HER-2/*neu*-overexpressing breast and ovarian cancer xenografts were responsive to different classes of chemotherapeutic drugs compared to control-treated xenografts with no statistically significant differences between HER-2/*neu*-overexpressing and non-overexpressing xenografts. We found no instance in which HER-2/*neu*-overexpressing xenografts were rendered more sensitive to chemotherapeutic drugs *in vivo*. HER-2/*neu*-overexpressing xenografts consistently exhibited more rapid regrowth than control xenografts following initial response to chemotherapy suggesting that a high rate of tumor cell proliferation rather than intrinsic drug resistance may be responsible for the adverse prognosis associated with HER-2/*neu* overexpression in human cancers.

Keywords: HER-2/*neu* (c-*erb*B-2); breast cancer; ovarian cancer; drug resistance; chemotherapy

Introduction

The human HER-2/*neu* (c-*erb*B-2) proto-oncogene encodes a 185 kD transmembrane receptor tyrosine kinase which is homologous to, but distinct from, the epidermal growth factor receptor (EGFR) as well as other members of the type I receptor tyrosine kinase family (i.e. HER-3 and HER-4). Sequence identity between members of this receptor family in their extracellular, and intracellular tyrosine kinase domains is 40–60% and 60–80%, respectively (Rajkumar and Gullick, 1994). Amplification of the HER-2/*neu* gene occurs in ~25–30% of human breast and ovarian cancers resulting in overexpression of the gene product, and this molecular alteration, when present, is an independent predictor of both relapse-free and overall survival in these diseases (Pauletti *et al.*, 1996; Slamon *et al.*, 1987). In breast cancer, overexpression of the HER-2/*neu* gene has been associated with a number of other adverse prognostic factors including: advanced pathologic stage (Seshadri *et al.*, 1993), number of axillary lymph node metastasis (Slamon *et al.*, 1987), absence of estrogen and progesterone receptors (Quenel *et al.*, 1995; Querzoli *et al.*, 1990; Barbareschi *et al.*, 1992), increased S-phase fraction (Borg *et al.*, 1991; Anbazhagan *et al.*, 1991), DNA ploidy (Stal *et al.*, 1994; Lee *et al.*, 1992), and high nuclear grade (Berger *et al.*, 1988; Poller *et al.*, 1991). A role for the HER-2/*neu* alteration in metastasis has also been suggested given the increased occurrence of visceral metastasis (Kallioniema *et al.*, 1991) and higher incidence of micrometastatic bone marrow disease (Pantel *et al.*, 1993) in patients with HER-2/*neu* overexpression. In addition, expression of HER-2/*neu* has prognostic significance in patients with gastric (Yonemura *et al.*, 1991), endometrial (Berchuck *et al.*, 1991; Hetzel *et al.*, 1992; Lukes *et al.*, 1994; Saffari *et al.*, 1995), and salivary gland cancers (Semba *et al.*, 1985; Press *et al.*, 1994). The exact role alteration of HER-2/*neu* receptor expression plays in the pathogenesis of these cancers remains unclear.

Retrospective data from two large clinical trials in breast cancer suggests an association between HER-2/*neu* overexpression and resistance to chemotherapy. Results from the Intergroup Study 0011 (Allred *et al.*, 1992) and the International (Ludwig) Breast Cancer Study Group (Gusterson *et al.*, 1992) led investigators to conclude that node-negative breast cancer patients whose tumors contain HER-2/*neu* overexpression have a less favorable prognosis due to a lack of response to adjuvant cyclophosphamide (CPA), methotrexate (MTX), and 5-fluorouracil (5-FU)-based chemotherapy (CMF). In addition, in a study of 68 patients with advanced breast cancer, Wright and colleagues reported a shortened survival for patients with HER-2/*neu* overexpression who were treated with mitoxantrone despite the fact that response rates between HER-2/*neu*-overexpressing and non-overexpressing tumors were similar, 50% *vs* 58%, respectively (Wright *et al.*, 1992). A study of HER-2/*neu* overexpression in epithelial ovarian cancer demonstrated that patients whose tumors had the alteration were more likely to fail chemotherapy with CPA and carboplatin (CBDCA) (Felip *et al.*, 1995). Conversely, in a clinical series reviewed by Klijn *et al.* patients with

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metastatic breast cancer and amplification of the HER-2/*neu* gene had a superior response to CMF chemotherapy (75%) compared to patients without HER-2/*neu* amplified tumors (45%) and the median length of progression-free survival from the start of chemotherapy was superior in patients whose tumors exhibited amplification (Berns *et al.*, 1995; Klijn *et al.*, 1993). Recently, data from the Cancer and Leukemia Group-B demonstrated that node-positive breast cancer patients with HER-2/*neu* overexpression derived a benefit from doxorubicin (DOX)-based adjuvant chemotherapy which is dose-dependent indicating that HER-2/*neu* overexpression may be associated with an increased response to DOX (Muss *et al.*, 1994). In composite, the clinical data to date are somewhat contradictory and do not adequately define what role, if any, HER-2/*neu* overexpression plays in chemotherapy response. Moreover, there is little experimental data to address this potentially important question. In one study evaluating *in vitro* chemosensitivity in HER-2/*neu*-transfected MCF7 breast carcinoma cells, no significant difference in response to either 5-FU or DOX was seen, while HER-2 overexpression was associated with a 2–4-fold increase in resistance to cisplatin (CDDP) (Benz *et al.*, 1992). In another study, HER-2/*neu* transfection of MDA-MB-435 cells conferred resistance to paclitaxel (TAX) via an *mdr*-1-independent mechanism (Yu *et al.*, 1996). *In vitro* studies of lung cancer cell lines demonstrated an association between HER-2/*neu* expression levels and intrinsic chemoresistance to six different chemotherapeutic drugs (Tsai *et al.*, 1993), and transfection of HER-2/*neu* cDNA into one lung cancer cell line resulted in an increase in drug resistance (Tsai *et al.*, 1995).

In an attempt to further define the effect of HER-2/*neu* overexpression on sensitivity to chemotherapeutic drugs in human breast and ovarian cancers, we introduced a full-length, human HER-2/*neu* cDNA, via a retroviral expression vector, into four different breast cancer cell lines: MCF7, MDA-MB-231, MDA-MB-435 and BT-20, and two different ovarian carcinoma cell lines: 2008 and Caov-3. All of the parental cell lines used for this study contain a single copy of the HER-2/*neu* gene and express basal levels of the gene product while the matched HER-2/*neu* retroviral transfectants overexpress the gene. Dose-response curves using seven different classes of chemotherapeutic agents were constructed for the HER-2/*neu*-overexpressing cell lines as well as their mock-transfected parental controls. The rationale for this experimental approach was to allow direct comparison of genetically identical parent/daughter cells which differ only in that one member of the pair overexpresses the human HER-2/*neu* gene. This approach was taken to circumvent the difficulty of comparing cell lines derived from separate sources which may inherently differ in characteristics other than HER-2/*neu* overexpression which could impact on drug sensitivity. The rationale for evaluating more than one cell line representing each of these two human malignancies is to avoid the possibility that any given observation could be unique to an individual cell line rather than being representative of a more generic biologic effect associated with HER-2/*neu* overexpression. Finally, to avoid the possibility that any observed effects might be restricted to an *in vitro* setting and because monolayer cell culture assays may not detect important multicellular mechanisms of drug resistance (Kerbel *et al.*, 1994; Kerbel, 1995), chemosensitivity was tested *in vivo* for breast and ovarian cancer parent/daughter xenografts in an athymic mouse model.

Results

*Characterization of human breast and ovarian cancer cells engineered to overexpress the HER-2/*neu *gene*

A full-length HER-2/*neu* cDNA was introduced via retroviral vector into a panel of human breast and ovarian carcinoma cells which are known to have a single copy of the HER-2/*neu* gene and to express 'normal' levels of the gene product. Breast cell lines BT-20 and MDA-MB-435 were established from previously untreated patients making them less likely to have treatment-induced chemotherapeutic drug resistance while the MCF7 cell line was established from a patient with prior radiation and hormonal therapy and the MDA-MB-231 cell line was derived from a patient previously treated with multidrug chemotherapy (5-FU, CPA, DOX, MTX, and prednisone). The ovarian carcinoma cell line 2008 was established from a patient who had not had prior chemotherapy, whereas the Caov-3 cell line was derived from a patient whose tumor had been exposed to prior 5-FU, DOX, and CPA *in vivo*. This spectrum of cell lines allows for response data representative of a diverse group of human breast and ovarian cancers. HER-2/*neu*-engineered and control cells were identically infected using a neomycin phosphotransferase-based vector which either contained, or did not contain, a full-length HER-2/*neu* cDNA. Retroviral infectants were selected for neomycin resistance and subjected to fluorescence activated cell sorting (FACS) analysis for detection of the $p185^{HER-2}$ protein. Western blot analysis confirmed a marked increase in $p185^{HER-2}$ expression in cells engineered to overexpress the gene relative to mock (NEO)-infected controls (Figure 1a and b). SK-BR-3 human breast carcinoma cells and SK-OV-3 human ovarian carcinoma cells naturally overexpress the HER-2 receptor and were included in these studies for comparison of non-manipulated overexpressing

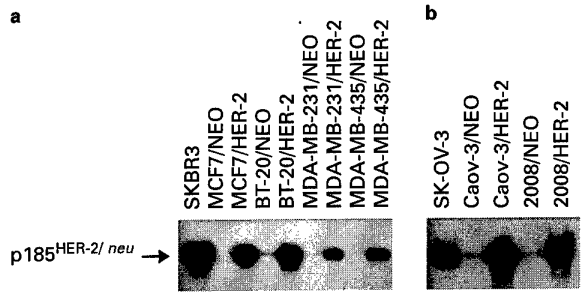

Figure 1 Western blot analysis of HER-2/*neu*- and mock (NEO)-vector infected breast (**a**) and ovarian (**b**) carcinoma cell lines demonstrating high-level expression of $p185^{HER-2}$ in transfected cell lines. SK-BR-3 breast cells and SK-OV-3 ovarian cells have native amplification/overexpression of the HER-2/*neu* gene and are shown as positive controls

cells to the engineered cells. The levels of HER-2/*neu* overexpression in the engineered cells are comparable to, but do not exceed, the levels found in actual human tumors circumventing the possibility that any observed biologic changes are artifacts of levels of overexpression which do not occur in nature. As a measure of functional activity of $p185^{HER-2}$, the phosphorylation state of $p185^{HER-2}$ was assessed using immunoblotting techniques. Protein lysates from each of the transfected cell lines were subjected to immunoprecipitation with a $p185^{HER-2}$ specific monoclonal antibody. These experiments were performed on cell lines both with and without prior exposure to heregulin B-1, a growth factor ligand cloned on the basis of its ability to induce tyrosine phosphorylation of $p185^{HER-2}$ through the formation of HER-2/HER-3 and/or HER-2/HER-4 heterodimeric complexes (Sliwkowski *et al.*, 1994; Plowman *et al.*, 1993). The resulting immunoprecipitates were then resolved by polyacrylamide gel electrophoresis (SDS–PAGE) and probed with an anti-phosphotyrosine antibody (Figures 2a and 3a). These results indicate that HER-2/*neu* cDNA transfection results in expression of a $p185^{HER-2}$ protein which is either constitutively tyrosine phosphorylated or can be phosphorylated on exposure to heregulin B-1 in each of the breast cell lines with the exception of MDA-MB-231 (Figure 2a). Similarly, ovarian Caov-3/HER-2 cells exhibited heregulin-induced tyrosine phosphorylation of $p185^{HER-2}$ while 2008/HER-2 cells did not (Figure 3a). In Figures 2b and 3b the same blots from Figures 2a and 3a have been probed with the same anti-$p185^{HER-2}$ antibody used for the immunoprecipitations. These results confirm overexpression of $p185^{HER-2}$ protein in the HER-2/*neu*-transfected cell lines, and in addition, demonstrate that exposure of the mock-vector (NEO) transfected cell lines to heregulin B-1 in most cases resulted in tyrosine phosphorylation as well as down-regulation of $p185^{HER-2}$ expression (Figures 2b and 3b). The relative degree of heregulin induced tyrosine phosphorylation of $p185^{HER-2}$ correlated with the expression level of HER-3 in these cells. For example, MCF7 cells have 2.5×10^4 HER-3 molecules per cell whereas MDA-MB-231 and 2008 cells have only 1.4×10^3, and 1.0×10^3 HER-3 molecules per cell, respectively by quantitative ELISA (Aguilar *et al.* manuscript in preparation). HER-4 expression levels are very low, $< 10^3$ molecules/cell, relative to HER-2 or HER-3 in this panel of cell lines, therefore heregulin-induced HER-2 phosphorylation appears to be predominantly influenced by the abundance of HER-2/HER-3 heterodimers in these cells. Having successfully engineered the breast and ovarian cells to overexpress $p185^{HER-2}$, we next evaluated the effects of overexpression on their sensitivity to chemotherapeutic drugs *in vitro* and *in vivo*.

Effect of HER-2/neu *overexpression on sensitivity of human breast and ovarian cells to chemotherapeutic agents* in vitro

The effects of HER-2/*neu* overexpression in human breast and ovarian carcinoma cell lines on sensitivity to a variety of chemotherapeutic agents was determined *in vitro*. The effective dose range for each drug (IC_{10}–IC_{90}) was identified using a range of ten different doses, each tested in quintuplicate. To assure accuracy and reproducibility, all sets of *in vitro* assays were repeated at least two times. This assay yielded 4-parameter, sigmoidal curve fits with correlation coefficients ranging from 0.938–0.999. Differences between dose-response curves were assessed using 2-factor analysis of variance (ANOVA) of data points which fell between the IC_{20} and IC_{80}. Representative data from these experiments

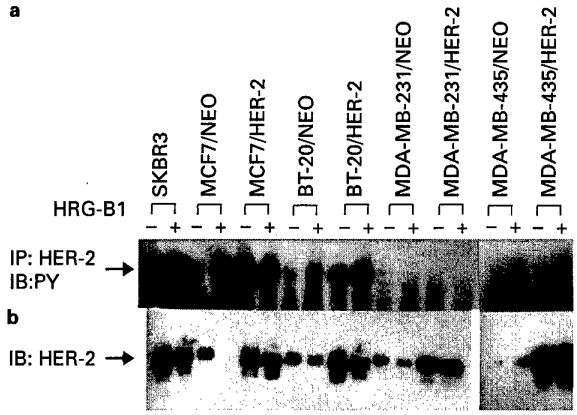

Figure 2 To demonstrate the phosphorylation state of $p185^{HER-2}$ in HER-2/*neu*-transfected breast carcinoma cell lines, anti-phosphotyrosine immunoblots were performed following immunoprecipitation with a monoclonal anti-$p185^{HER-2}$ antibody both in the presence (+) or absence (−) of recombinant heregulin B-1 (**a**). The same blot is reprobed with anti-$p185^{HER-2}$ (**b**). These data demonstrate constitutive tyrosine phosphorylation of $p185^{HER-2}$ in SKBR3, MCF7/HER-2 and BT-20/HER-2 even in the absence of heregulin B-1. In mock (NEO)-transfected MCF7 and BT-20 cells, heregulin B-1 induced both an increase in $p185^{HER-2}$ tyrosine phosphorylation (**a**) and downregulation of $p185^{HER-2}$ expression (**b**). MDA-MB-231 cells exhibited neither basal nor heregulin-induced tyrosine phosphorylation of $p185^{HER-2}$ despite high expression levels of the protein

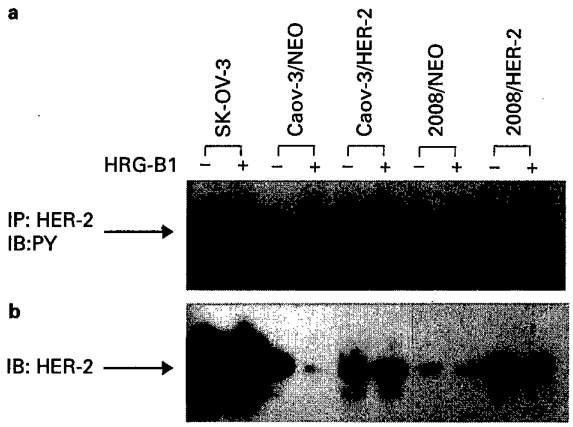

Figure 3 Anti-phosphotyrosine immunoblot of HER-2/*neu*- or mock (NEO)-transfected ovarian carcinoma cells following immunoprecipitation with an anti-$p185^{HER-2}$ specific monoclonal antibody either in the presence (+) or absence (−) of exogenous recombinant heregulin B-1 (**a**). The same blot is reprobed with anti-$p185^{HER-2}$ (**b**). The data demonstrate an increase in $p185^{HER-2}$ tyrosine phosphorylation and downregulation of $p185^{HER-2}$ expression on exposure to heregulin B-1 in Caov-3/NEO cells. Caov-3/HER-2 cells demonstrate both basal and heregulin-induced tyrosine phosphorylation of $p185^{HER-2}$ whereas 2008/HER-2 have neither increased basal or heregulin-induced $p185^{HER-2}$ phosphorylation despite overexpression of the protein

are shown in Tables 1 and 2. These data include the $IC_{50} \pm$ one standard deviation and the significance level for differences between control (NEO) and HER-2-engineered cell lines. Introduction of neomycin phosphotransferase gene via the NEO control vector and selection in neomycin resulted in no change in chemosensitivity in MCF7 cells (data not shown) indicating that neomycin resistance does not confer cross-resistance to chemotherapeutic agents *in vitro*. Clinically achievable peak plasma levels of chemotherapeutic drugs from standard dosing schedules used in humans are shown for reference in Table 1.

HER-2/*neu* overexpression in MCF7 breast carcinoma cells resulted in a 2.5-fold decrease in sensitivity to the platinum analog CBDCA, as well as a twofold decrease in 5-FU sensitivity. Conversely, a twofold increase in sensitivity to TAX was noted while no change in sensitivity to the other four drugs tested was found (Table 1). These results are similar to those reported by Benz *et al.* who noted a 2–3-fold decrease in sensitivity to CDDP but no change in sensitivity to DOX or 5-FU in MCF7 cells which overexpress HER-2/*neu* (Benz *et al.*, 1992). In contrast, MDA-MB-231/HER-2 cells were rendered more sensitive to four of the seven drugs tested (Table 1). This increase in sensitivity ranged from 1.4-fold for thiotepa (TSPA) to >100-fold for TAX. The BT-20/HER-2 cells were also 2–4-fold more sensitive to TSPA and 5-FU, but like MCF7/HER-2 cells, they were more resistant to platinum compounds. Lastly, MDA-MB-435/HER-2 cells exhibited no change in chemosensitivity to any of the seven classes of chemotherapeutic agents tested. Among the ovarian carcinoma cell lines, Caov-3/HER-2 cells were slightly more sensitive to DOX and vinblastine (VBL) compared to Caov-3/NEO; however, HER-2/*neu* overexpression in 2008 cells resulted in a threefold and 7.5-fold increase in resistance to CBDCA and TSPA, respectively (Table 2). These results indicate that HER-2/*neu* overexpression does not produce any consistent or predictable change in drug sensitivity profiles *in vitro* across the various cell lines tested and underscore the necessity of evaluating more than one cell line prior to drawing general conclusions on the effect of this alteration on chemotherapeutic response in human cancer cells. Moreover, the differences in chemosensitivity patterns among the HER-2/*neu*-transfected cell lines did not appear to correlate with basal or heregulin B-1-induced tyrosine phosphorylation of $p185^{HER\text{-}2}$. Despite the fact that chemosensitivity in HER-2/*neu*-overexpressing cells was cell line specific, some trends did emerge from the data. HER-2/*neu*-overexpression had no major effect on sensitivity to DOX in any of the six cell lines tested with the exception of Caov-3/HER-2 cells where it was associated with a small (0.5 μM to 0.3 μM) but statistically significant increase in sensitivity. Similarly, HER-2/*neu* overexpression had minimal effects on response to etoposide (VP-16) with only one cell line, MDA-MB-231, exhibiting a slight increase in sensitivity after transfection with HER-2/*neu*. Increased resistance to platinum analogs was observed in three of the six cell lines with HER-2/*neu* overexpression compared to their controls. Finally, when agents which interfere with microtubule formation (VBL and TAX) were studied, three of six HER-2/*neu*-overexpressing cell lines demonstrated an increase in sensitivity.

Effect of HER-2/neu *expression on chemosensitivity of breast and ovarian xenografts* in vivo

To further evaluate and expand drug sensitivity studies associated with HER-2/*neu* overexpression, we developed an *in vivo* chemotherapeutic drug sensitivity assay which utilized serial measurements

Table 1 Effect of HER-2/*neu* overexpression on sensitivity of human breast cells to chemotherapeutic agents *in vitro*[a]

	CDDP (μM)[b]	*DOX* (μM)	*5-FU* (μM)	*TAX* (nM)	*TSPA* (μM)	*VBL* (nM)	*VP-16* (μM)
MCF7/NEO	19.1±5.0	0.39±0.03	10.3±3.4	20.2±3.9	78.5±13.0	0.93±0.09	16.0±1.0
MCF7/HER-2	48.4±7.8*	0.34±0.07	22.5±6.0***	9.6±9.6**	85.2±9.6	1.1±0.05	14.0±3.0
MDA-MB-435/NEO	13.0±1.3	0.6±0.09	7.6±0.7	1.2±0.1	75.6±4.2	0.4±0.02	2.7±0.2
MDA-MB-435/HER-2	13.3±2.3	0.6±0.07	9.9±1.2	1.2±0.05	77.1±2.1	0.3±0.02	3.2±0.2
MDA-MB-231/NEO	21.6±6.0	0.3±0.03	50.0±9.0	14.6±1.5	238.3±17.4	19.0±2.5	10.2±0.5
MDA-MB-231/HER-2	20.3±4.0	0.2±0.05	44.3±12.0	0.08±0.05***	167.0±7.4*	1.2±1.0***	3.4±0.5**
BT-20/NEO	3.6±0.3	0.17±0.03	130.0±20.2	5.8±1.2	228.3±25.0	0.2±0.06	15.1±1.2
BT-20/HER-2	25.7±2.0***	0.15±0.02	32.0±7.0***	4.2±1.2	117.8±20.6**	0.3±0.1	12.2±0.2
[Peak Plasma] Reference	30 (Gormley *et al.*, 1979)	5.6 (Robert *et al.*, 1982)	1000 (MacMillan *et al.*, 1978)	940 (Wiernik *et al.*, 1987)	10.6 (Cohen *et al.*, 1986)	400 (Nelson *et al.*, 1980)	50 (D'Incalci *et al.*, 1982)

[a]*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. [b]CBDCA substituted for CDDP for MCF7/NEO and MCF7/HER-2. Peak plasma concentration of CBDCA is 50 μM (Harland *et al.*, 1984). Data shown are IC_{50} values for each drug. Error is reported as ±one standard deviation. The peak plasma levels of each drug achievable in humans with standard dosing schedules are shown for reference

Table 2 Effect of HER-2/*neu* overexpression on sensitivity of human ovarian cells to chemotherapeutic agents *in vitro*[a]

	CDDP (μM)[b]	*DOX* (μM)	*5-FU* (μM)	*TAX* (nM)	*TSPA* (μM)	*VBL* (nM)	*VP-16* (μM)
Caov-3/NEO	20.0±1.3	0.5±0.05	16.0±3.8	24.8±6.2	80.9±1.6	1.1±0.1	1.2±0.2
Caov-3/HER-2	19.1±0.3	0.3±0.04*	15.3±1.5	21.7±3.0	85.6±4.8	0.5±0.03*	1.2±0.3
2008/NEO	1.3±0.3	0.06±0.007	3.6±0.5	1.5±0.2	4.9±1.8	1.0±0.3	0.5±0.03
2008/HER-2	3.9±0.3***	0.06±0.01	5.3±0.9	1.6±0.2	37.0±7.4***	1.6±0.7	0.4±0.05

[a]*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. [b]CBDCA substituted for CDDP for 2008/NEO and 2008/HER-2. Data indicate IC_{50} values for each drug. Experimental error is reported as ±one standard deviation

of subcutaneous human tumor xenografts growing in athymic mice. For the *in vivo* studies, human breast (MCF7) and ovarian (2008) carcinoma cells were selected for testing because of their predictable tumor formation in athymic mice. Immunohistochemical analysis of sections from these tumors and Western blot analysis from cell lines derived from these xenografts confirmed that the relative expression level of HER-2/*neu* was maintained during the course of the study (data not shown). Overexpression of HER-2/*neu* in MCF7 breast carcinoma cells resulted in a significant change in their *in vivo* growth characteristics (Figure 4). By day 50, MCF7/HER-2 tumors were 2.7-fold larger than MCF7/NEO tumors ($P=0.0001$). At the onset of chemotherapy administration, animals were assigned to treatment groups such that initial tumor volumes were the same in each group (55 ± 4 mm^3). Because the MCF7/NEO xenografts have a significant difference in inherent growth rate compared to MCF7/HER-2 xenografts, the ratio of chemotherapy-treated to untreated control tumor volume (T/C ratio) was calculated for each tumor. The maximum response to chemotherapy, defined as the point at which the T/C ratio was at a minimum, was determined for each individual tumor. The maximum drug responses for the MCF7/NEO xenografts were then directly compared to responses found in the MCF7/HER-2 xenografts.

In the human breast cancer xenograft model, all five drugs tested resulted in significant responses for both MCF7/NEO and MCF7/HER-2 tumors compared to their respective untreated control tumors ($P<0.05$) indicating that HER-2/*neu*-transfected MCF7 xenografts maintain sensitivity to these chemotherapeutic drugs *in vivo* (Figure 5). The mean time to point of maximum response was 17 ± 5 days and was independent of the drug tested or tumor type (i.e. NEO *vs* HER-2). Tumor regrowth following day 21 uniformly occurred indicating a lack of prolonged response to the initial treatment. Of note was the fact that there was a significant difference in regrowth rates following responses to chemotherapy when comparing MCF7/NEO to MCF7/HER-2 tumors. The mean tumor doubling time following chemotherapy was 14.6 days for MCF7/HER-2 tumors compared to 23.8 days for MCF7/NEO tumors ($P=0.0001$). This demonstrates that HER-2/*neu*-overexpressing tumors maintain their proliferative advantage following exposure to chemotherapy *in vivo*. The T/C ratios at the point of maximum response are represented graphically by box plots (Figure 5). Treatment with DOX resulted in significant responses for both MCF7/NEO and MCF7/HER-2 tumors compared to their respective untreated control groups (Figure 5a). The difference in the magnitude of maximum response to DOX between MCF7/NEO and MCF7/HER-2 tumors was not statistically significant ($P=0.13$). Treatment with CDDP also resulted in significant responses for both MCF7/NEO and MCF7/HER-2 tumors and again mean CDDP-treated T/C ratios were not significantly different at the point of maximum response (Figure 5b, $P=0.12$). Similarly, treatment with 5-FU resulted in significant responses compared to controls for both MCF7/NEO and MCF7/HER-2 tumors (Figure 5c); but the difference in response between MCF7/NEO and MCF7/HER-2 was not statistically significant ($P=0.12$). Treatment with TAX also resulted in significant responses for MCF7/NEO and MCF7/HER-2 tumors compared to vehicle-treated controls. Mean TAX-treated T/C ratios at maximum response were 0.19 ± 0.09 and 0.30 ± 0.18 for MCF7/NEO and MCF7/HER-2 tumors, respectively (Figure 5d), and this difference was marginally significant ($P=0.09$). Finally, response to treatment with TSPA was significant for both MCF7/NEO and MCF7/HER-2 tumors compared to control (Figure 5e), but there was no significant difference between response of MCF7/NEO xenografts compared to MCF7/HER-2 xenografts in response to TSPA ($P=0.17$). Additional analysis in a 2-factor ANOVA model failed to

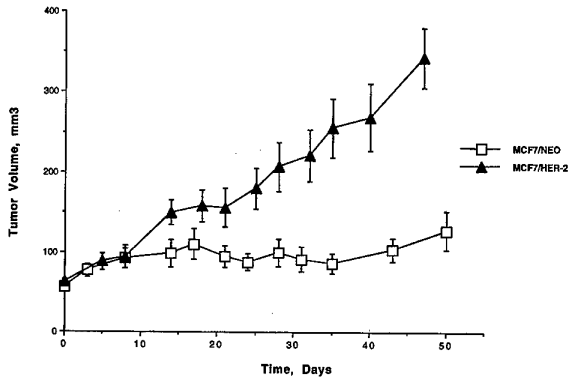

Figure 4 Tumorigenicity of HER-2/*neu*, or control (NEO) vector-infected human breast (MCF7) s.c. xenografts in female athymic mice ($n=13-14$/group). Error bars indicate standard error. MCF7/HER-2 xenografts (▲) have a significant growth advantage over MCF7/NEO (□) ($P=0.0001$) *in vivo*. Mice in this experiment were treated with a vehicle control solution i.p. beginning on day 0 (12 days status post xenograft inoculation), at which time objectively measurable xenografts had formed

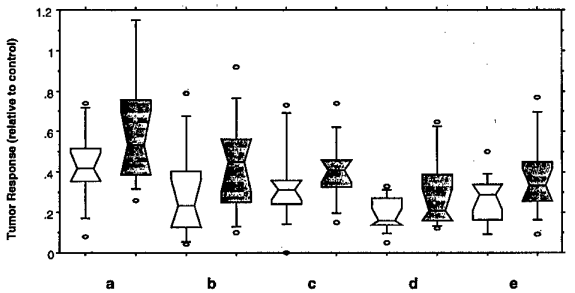

Figure 5 Box Plots illustrating tumor response (relative to control) for MCF7/NEO (unshaded boxes) and MCF7/HER-2 (shaded boxes) xenografts ($n=12-14$ per group) in response to treatment with: (**a**) DOX (5 mg/kg), (**b**) CDDP (5 mg/kg), (**c**) 5-FU (100 mg/kg), (**d**) TAX (15 mg/kg × 3), and (**e**) TSPA (5 mg/kg × 3). Error bars indicate 10th to 90th percentiles, boxes indicate 25th to 75th percentiles, and notches indicate 95% confidence intervals. Group mean T/C ratios and significance levels (Mann-Whitney U test) for differences between MCF7/NEO and MCF7/HER-2 are as follows:

Group means	MCF7/NEO	MCF7/HER-2	Significance Level
a	0.43	0.62	$P=0.13$
b	0.30	0.44	$P=0.12$
c	0.33	0.41	$P=0.12$
d	0.19	0.30	$P=0.09$
e	0.27	0.38	$P=0.17$

demonstrate significant differences in the magnitude of response between MCF7/NEO and MCF7/HER-2 xenografts to any chemotherapeutic agent tested over the time period during which responses were seen.

Unlike MCF7 cells, the ovarian carcinoma cells 2008/HER-2 had only a small growth advantage over 2008/NEO cells (Figures 6a–c, vehicle controls). In this model, both the 2008/NEO and 2008/HER-2 xenografts were refractory to treatment with DOX using two different treatment schedules (5 mg/kg on day 1 or 3 mg/kg on days 1 and 14, data not shown). Higher doses of DOX resulted in substantial toxicity. Similarly, VP-16 at a dose of 25 mg/kg on days 0, 3, and 7 had no effect on 2008/NEO or 2008/HER-2 tumor growth during the 21 day observation period. A dose of 50 mg/kg on day 0 and day 3 did result in a significant response compared to untreated control tumors by day 6 (data not shown), however there was no response difference between 2008/NEO and 2008/HER-2 tumors, and the higher dose of VP-16 resulted in substantial mortality beyond day 6. Treatment of ovarian 2008 tumors with CDDP resulted in significant responses by day 6 at which time tumor volumes of CDDP-treated tumors were 37% of controls and significant differences were maintained during a 21 day observation period (Figure 6a). There was no difference, however, in the degree of response between 2008/NEO and 2008/HER-2 ovarian xenografts, thus the threefold shift in IC_{50} suggesting platinum resistance in the HER-2/*neu*-overexpressing cells *in vitro* was not observed *in vivo*. Treatment of ovarian 2008 NEO and HER-2 tumors with TAX resulted in a 58% reduction in tumor volume compared to control which was apparent at day 6. However, there was no difference in response when comparing 2008/NEO and 2008/HER-2 tumors indicating that HER-2/*neu* overexpression in these cells had no impact on sensitivity to TAX *in vivo* (Figure 6b). Treatment of ovarian 2008 xenografts with TSPA also resulted in a significant response compared to untreated control tumors. For this drug, a significant difference between TSPA-treated 2008/NEO and 2008/HER-2 tumors did emerge by day 21 with TSPA-treated 2008/HER-2 tumors measuring 100% larger than TSPA-treated 2008/NEO tumors ($P=0.002$) (Figure 6c). Moreover, this result paralleled the *in vitro* results where a 7.5-fold increase in resistance to TSPA was noted in 2008 cells overexpressing HER-2/*neu*. This difference, however, appeared to be due to more rapid tumor regrowth for 2008/HER-2 xenografts following response to chemotherapy rather than intrinsic resistance to TSPA. In fact, at the time of maximal response to TSPA (day 10), there was no significant difference between 2008/NEO and 2008/HER-2 xenografts ($P=0.17$). These data paralleled the results seen with MCF7/HER2 xenografts where rapid tumor regrowth occurred following response to chemotherapy *in vivo*.

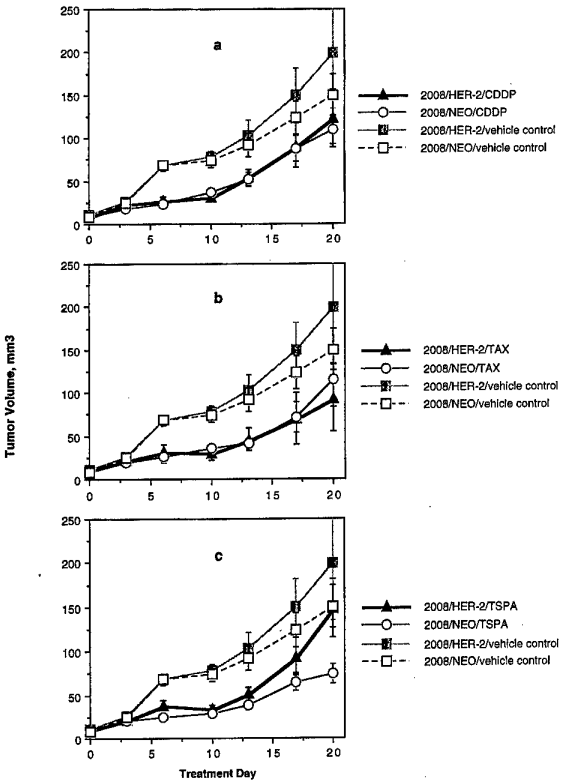

Figure 6 Response of human ovarian xenografts 2008/NEO and 2008/HER-2 to treatment with (**a**) CDDP (5 mg/kg), (**b**) TAX (15 mg/kg × 3), and (**c**) TSPA (5 mg/kg × 3) in female athymic mice. Injection of a single cycle of these three drugs resulted in significant responses compared to a vehicle control for both 2008/NEO and 2008/HER-2 xenografts; however, the magnitude of response was not significantly different for 2008/NEO compared to 2008/HER-2 xenografts. The growth rate of TSPA-treated 2008/HER-2 xenografts (**c**) was significantly greater than 2008/NEO xenografts ($P=0.002$) following an initial response to TSPA

Discussion

The involvement of some oncogenes in the development of chemotherapeutic drug resistance is suggested by experimental data demonstrating increased expression of c-*fos*, c-*myc*, and c-H-*ras* gene transcripts in cisplatin-resistant human ovarian carcinoma cells (Scanlon *et al*., 1989). In transfection studies, c-*myc* expression was subsequently shown to result in an increase in resistance of Friend erythroleukemia cells to CDDP (Sklar and Prochownik, 1991) and the acquisition of a multidrug-resistant phenotype in NIH3T3 cells (Niimi *et al*., 1991). Transfection of c-H-*ras* oncogene into NIH3T3 cells has also been shown to induce resistance to CDDP (Isonishi *et al*., 1991) and in one study both *ras* and *trk*-transformed NIH3T3 fibroblasts were less sensitive to CDDP and DOX compared to parental NIH3T3 cells (Peters *et al*., 1993). More studies show an indirect relationship between oncogene expression and drug resistance. Introduction of v-H-*ras* or v-H-*raf* into rat hepatocytes results in increased expression of *mdr*-1 (P-glycoprotein) which is associated with multidrug resistance (Burt *et al*., 1988). Marked increases in EGFR expression have been detected in several different cell types selected for resistance to natural-product anticancer drugs such as DOX, vincristine, and actinomycin-D (Meyers *et al*., 1986; Nuti *et al*., 1991; Dickstein *et al*., 1993); and increased resistance to DOX, VBL, CDDP and 5-FU has been reported in ZR75B human breast cancer cells transfected with EGFR (Dickstein *et al*., 1995). In addition, ligands to

and antibodies directed against EGFR have been shown to modulate sensitivity to chemotherapeutic drugs (Aboud-Pirak *et al.*, 1988; Christen *et al.*, 1990), and 'cross talk' between EGFR and P-glycoprotein is suggested by increases in P-glycoprotein phosphorylation in actinomycin-D-resistant Chinese hamster lung cells treated with epidermal growth factor (Meyers *et al.*, 1993).

Data from several clinical trials indicate a possible association between HER-2/*neu* overexpression and chemosensitivity, leading to speculation that overexpression of this proto-oncogene may also be relevant in predicting chemotherapeutic response. However, the potential role of HER-2 receptor overexpression in the development of chemotherapeutic drug resistance remains unclear for at least three reasons: (1) the conflicting nature of the results published from the various clinical trials to date; (2) the paucity of experimental data describing the effects of HER-2/*neu* overexpression on drug sensitivity; and (3) the fact that studies done thus far using transfection strategies are restricted to single cell lines and/or do not address chemotherapeutic responses *in vivo*. Assessing the generic role of a given gene in the acquisition of chemotherapeutic drug resistance using typical transfection and selection strategies in a single cell line may be problematic due to inherent differences in chemosensitivity of cell lines derived from different sources. In addition, following transfection and selection, individual subclones may possess varying degrees of sensitivity to chemotherapeutic agents which are random. Potential non-generic or cell line specific changes in chemosensitivity associated with HER-2/*neu* overexpression were avoided in the current studies by using multiple human cell lines to construct parent/daughter pairs which differ only in their HER-2/*neu* expression level, circumventing the possibility that consistent observations across cell lines would be attributable to such effects. Moreover, two different epithelial cell types were analysed both *in vitro* and *in vivo* to significantly decrease the chances that a consistently observed change might be due to phenomena unrelated to HER-2/*neu* overexpression but rather to a given epithelial type or assay method. We also sorted cells following transfection with HER-2/*neu* using FACS which results in collection of a pooled population (approximately 5×10^5) of HER-2/*neu*-overexpressing cells rather than individual subclones.

Using these approaches we found that HER-2/*neu* overexpression alone was not sufficient to induce intrinsic, pleomorphic drug resistance in human breast or ovarian carcinoma cell lines and did not result in any consistent or predictable changes in chemosensitivity profiles in an *in vitro* cell proliferation assay. The changes in chemosensitivity which were observed were cell line specific and not generic across the cell lines tested. This is illustrated by the fact that overexpression of the HER-2/*neu* receptor in MDA-MB-435 breast carcinoma cells had no effect on chemosensitivity to any of the seven different classes of drugs tested, whereas HER-2/*neu*-overexpressing MDA-MB-231 cells were rendered more sensitive to four of the seven drugs, and ovarian 2008 cells were rendered more resistant to two of the seven drugs tested. There are several potential reasons why HER-2/*neu* transfection results in alterations in chemosensitivity profiles which are cell line dependent. First, a number of the cell lines used in this study were derived from tumors of patients who had prior exposure to chemotherapeutic agents and, as a result, may have already developed some degree of drug resistance. For example, Caov-3 cells are derived from a patient who had been exposed to combination chemotherapy and these cells are less sensitive to most of the drugs tested when compared to ovarian 2008 cells. Second, the effects of HER-2/*neu* transfection may be influenced by other genetic alterations within a given cell line. Support for this hypothesis was demonstrated in co-transfection studies of both HER-2/*neu* and mutated c-H-*ras* in which induction of *mdr*-1 expression and resulting P-glycoprotein activity was observed only after co-transfection with both HER-2/*neu* and c-H-*ras* while neither gene alone resulted in a multidrug resistant phenotype (Sabbatini *et al.*, 1994). Third, the drug sensitivity profile of a given cell line following HER-2/*neu* transfection and overexpression may depend on the cellular context in which HER-2/*neu* is overexpressed. For example, co-expression of other type 1 receptor tyrosine kinases within a cell may influence HER-2/*neu* activity and subsequent intracellular signaling via formation of specific class 1 heterodimeric receptor species (Sliwkowski *et al.*, 1994; Plowman *et al.*, 1993). We were able to demonstrate differences in basal and heregulin B-1-induced tyrosine phosphorylation of $p185^{HER-2}$ among the cell lines tested. To what degree co-expression of EGFR, HER-3, or HER-4 explain the differences in heregulin B-1-induced tyrosine phosphorylation of $p185^{HER-2}$ is the subject of ongoing investigations in our laboratory. It is clear from our results that some HER-2/*neu*-overexpressing cell lines exhibit shifts in drug sensitivity *in vitro* even in the absence of $p185^{HER-2}$ tyrosine phosphorylation (MDA-MB-231/HER-2 cells). Conversely, we found examples of cell lines which did exhibit heregulin B-1-induced tyrosine phosphorylation of $p185^{HER-2}$ and yet demonstrated no significant shifts in chemosensitivity either *in vitro* (MDA-MB-435/HER-2) or *in vivo* (MCF7/HER-2). Our data on response of MDA-MB-435/HER-2 cells to TAX appears to differ from data reported previously (Yu *et al.*, 1996). Our data are derived from a pooled population of HER-2/*neu*-transfected MDA-MB-435 cells whereas the data reported by Yu *et al.* is based on analysis of three subclones of MDA-MB-435/HER-2 cells. In addition, the shift in IC_{50} noted by Yu *et al.* occurred at TAX concentrations in the millimolar range which is above the peak serum concentration achievable in humans, whereas we report the IC_{50} of TAX on MDA-MB-435 cells to be 1.2 nanomolar. This apparent discrepancy may be explained by the different methodologies used to measure response to TAX–clonogenic assays used in the previous study *vs* monolayer cell proliferation assays used in the current study. Finally, some of the *in vitro* changes in chemosensitivity observed in this study may not be clinically relevant as they occur at drug concentrations which are well above the peak plasma levels achievable *in vivo*. The HER-2/*neu*-overexpressing breast carcinoma cells MDA-MB-231 and BT-20 appear to be more sensitive to TSPA but the shift in dose-response occurs at a drug concentration 20-fold higher than levels routinely achievable in humans.

HER-2/*neu* transfection resulted in decreased *in vitro* sensitivity to platinum analogs in three of the six human tumor cell lines tested. This observation is of interest in light of recent studies which show that some anti-HER-2 antibodies are capable of increasing sensitivity to platinum through a mechanism involving a decrease in DNA repair activity (Hancock *et al.*, 1991; Pietras *et al.*, 1994; Arteaga *et al.*, 1994). Except for a slight increase in sensitivity observed in Caov-3/HER-2 cells, HER-2/*neu* overexpression had no effect on sensitivity to DOX in any of the cell lines tested *in vitro*. Likewise HER-2/*neu* overexpression did not result in resistance to VP-16, TAX, or VBL which are known substrates for *mdr*-1 (Endicott and Ling, 1989). Furthermore, HER-2/*neu* overexpression did not substantially affect sensitivity to VP-16 which targets topoisomerase II (Liu, 1989). Topoisomerase II expression, however, has been found to be increased in ~12% of breast carcinomas with HER-2/*neu* overexpression and may be due to co-amplification of both genes owing to their close proximity on chromosome 17q (Smith *et al.*, 1993).

The shifts in dose-response curves secondary to HER-2/*neu* overexpression which were characterized *in vitro* did not result in parallel changes in chemosensitivity of the same cell lines *in vivo*. This is not surprising considering the limited capability of monolayer cell culture assays to recapitulate the complex microenvironment within a solid tumor in which physiologic, multicellular mechanisms of drug resistance are operative (Kobayashi *et al.*, 1993; Casciari *et al.*, 1994; Kerbel *et al.*, 1994; Kerbel, 1995). Furthermore, drug pharmacokinetics are markedly different *in vivo* compared to *in vitro*, and some degree of clonal selection may have unavoidably occurred *in vivo* causing differences in chemotherapy response compared to the pooled HER-2/*neu*-transfected clones *in vitro*. Xenografts resulting from HER-2/*neu*-overexpressing cells did respond, relative to control, to all of the chemotherapeutic drugs tested except in cases where control cell lines were inherently resistant to drug treatment such as 2008 ovarian tumors treated with DOX or VP-16 which fail to respond regardless of the presence or absence of HER-2/*neu* overexpression. In addition, the magnitude of response *in vivo* was similar for ovarian 2008/HER-2 and 2008/NEO xenografts for CDDP, TAX, and TSPA, indicating that HER-2/*neu* overexpression in this cell line did not induce intrinsic chemotherapeutic resistance to these drugs *in vivo*. However, 2008/HER-2 tumors demonstrated more rapid recovery following response to TSPA compared to 2008/NEO tumors. MCF7/HER-2 breast xenografts responded, relative to untreated controls, to each of five chemotherapeutic agents tested. The magnitude of response of MCF7/HER-2 tumors varied from 19% to 37% less than MCF7/NEO tumors for the five classes of cytotoxic drugs tested, suggesting the possibility of a slight increase in primary resistance to chemotherapy treatment *in vivo* for MCF7/HER-2 xenografts; however, this difference was not statistically significant. We found no instance in which xenografts resulting from HER-2/*neu*-overexpressing cell lines were rendered more sensitive to chemotherapeutic drugs *in vivo*. Therefore, in this experimental model, HER-2/*neu* overexpression alone is insufficient to confer increased sensitivity to DOX as has been hypothesized previously (Muss *et al.*, 1994). The MCF7 breast xenograft model, however, does demonstrate that HER-2/*neu*-transfected tumors are associated with a rapid rate of tumor regrowth following initial response to chemotherapy. Mean doubling time for tumor regrowth following response to chemotherapy was 14.6 days for MCF7/HER-2 tumors compared to 23.8 days for control-transfected tumors ($P = 0.0001$). These data suggest that the apparent lack of response to chemotherapy among patients with HER-2/*neu* positive tumors seen in some clinical trials may be due to rapid tumor regrowth of surviving tumor cells following initial response to chemotherapy rather than intrinsic chemotherapeutic drug resistance at the time of chemotherapy treatment.

The drug response phenotype is not static within solid tumors. New drug-resistant variants may emerge during chemotherapy treatment due to selection of pre-existing, drug-resistant clones within a heterogeneous tumor cell population, or through adaptive selection of spontaneously arising drug-resistant clones during the life of a tumor. Our data indicate that HER-2/*neu* overexpression alone in human breast and ovarian cancer cells is not sufficient to cause an intrinsic, pleotropic drug-resistant phenotype *in vitro*, nor does it significantly impair or enhance response to initial chemotherapy treatment *in vivo*. However, the growth stimulus afforded by overexpression of $p185^{HER\text{-}2}$ allows for rapid proliferation of any surviving cells following treatment with chemotherapy. This may in turn allow the emergence of acquired chemotherapeutic drug resistance through the processes of clonal or adaptive selection of resistant tumor cells. Experiments designed to test the effects of HER-2/*neu* overexpression on acquired rather than intrinsic drug resistance are underway in our laboratory. If, as our experimental models suggest, the adverse prognosis seen in patients whose tumors have amplification/overexpression of the HER-2/*neu* gene is due to rapid tumor cell proliferation rather than *de novo* resistance to chemotherapy, then maximizing reduction in tumor burden using more active agents and/or higher dose intensities may result in improved clinical response. This hypothesis is consistent with a recently published clinical trial (Muss *et al.*, 1994) and may mean that assessment of HER-2/*neu* status in malignant breast tissue is important in selecting treatment regimens for patients.

Materials and methods

Cell lines and cell culture

Human breast carcinoma cell lines MCF7, BT-20, MDA-MB-231, MDA-MB-435, and SK-BR-3, and human ovarian carcinoma cell lines Caov-3 and SK-OV-3, were obtained from American Type Culture Collection (Rockville, MD). Human ovarian 2008 cells were established from a patient with serious cystadenocarcinoma of the ovary (DiSaia *et al.*, 1972). All cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA).

*Transfection and overexpression of the human HER-2/*neu *gene in human breast and ovarian carcinoma cells*

Human breast and ovarian carcinoma cells with normal levels of HER-2/*neu* expression were transfected with a full-length cDNA of the human HER-2/*neu* gene. Introduction of HER-2/*neu* into human cells was accomplished using the replication defective retroviral expression vector pLXSN into which the HER-2/*neu* cDNA was ligated (Chazin *et al.*, 1992). The same pLXSN vector devoid of HER-2/*neu* sequences but containing the neomycin phosphotransferase gene was packaged in an identical fashion and was used to infect control cells. Breast and ovarian carcinoma cells were infected as previously described (Pietras *et al.*, 1994). Stably transfected cell lines were selected according to level of HER-2/*neu* expression using FACS with indirect immunofluorescence labeling mediated by the murine monoclonal anti-HER-2/*neu* antibody 4D5 (Genentech, Inc., South San Francisco, CA) and an anti-mouse IgG/FITC antibody (Caltag Laboratories, South San Francisco, CA). All cell lines were characterized for expression of HER-2/*neu* gene by Western blot analysis.

Western blot analysis

Cultured cells were washed in 137 mM NaCl solution containing 2.7 mM potassium chloride, 1.5 mM potassium phosphate and 8 mM sodium phosphate (Dulbecco's PBS, Gibco BRL, Gaithersburg, MD) and lysed at 4°C in 20 mM Tris, pH 8.0; 137 mM NaCl; 1% Triton X-100; 10% glycerol; 5 mM EDTA; 1 mM sodium orthovanadate; 1 mM phenylmethyl-sulfonylfluoride; leupeptin 1 μg/ml and aprotinin 1 μg/ml. Insoluble material was cleared by centrifugation at 10 000 *g* for 10 min. Protein was quantitated using BCA (Pierce Biochemicals, Rockford, IL), resolved by SDS–PAGE, and transferred to Immobilon-P (Millipore, Bedford, MA). The $p185^{HER-2}$ protein was detected by anti-c-*neu* (Oncogene Science, Uniondale, NY) using the ECL method (Amersham, Arlington Heights, IL).

*Tyrosine phosphorylation of the HER-2/*neu *receptor*

HER-2/*neu* and mock-vector transfected breast and ovarian cell lines were examined for phosphorylation of $p185^{HER-2}$ using SDS–PAGE, as described previously (Pietras *et al.*, 1995). In brief, cells were cultured to 80% confluence in 100 mm dishes in RPMI media containing 10% FCS. The cells were washed ×3 in PBS and then allowed to incubate in serum-free RPMI media for 24 h at 37°C. Recombinant heregulin B-1 (kindly provided by Dr M Sliwkowski, Genentech, Inc., S. San Francisco, CA) 10 mM or control solution was added and allowed to incubate for 5 min at 37°C. Cells were then washed in PBS and lysed using the conditions described above. Following protein quantitation, immunoprecipitations were performed by incubating 250 μg protein lysate with 5 μg/ml monoclonal anti-HER-2/*neu* antibody (Oncogene Science, Uniondale, NY) at 4°C overnight with gentle agitation. Protein A-agarose (BioRad, Richmond, CA) was added to precipitate the antigen-antibody complex and the immunoprecipitates were washed three times in lysis buffer prior to electrophoresis. Proteins were then transferred to Immobilon-P and immunoblotting was performed using monoclonal anti-phosphotyrosine antibody, PY20 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell proliferation assays

Aliquots of 5×10^3 cells were plated in quintuplicate in 96-well microdilution plates. Following cell adherence, experimental media containing either specific chemotherapeutic agents or control media was added. Serial twofold dilutions were performed to span the effective dose range for each drug. Representative drugs from seven different classes of chemotherapeutic agents were tested including: anthracycline antibiotics–DOX (Cetus Corporation, Emeryville, CA); antimetabolites–5-FU (Solo Park Laboratories, Inc., Elk Grove Village, IL); alkylating agents–TSPA (Lederle Laboratories, Pearl River, NY); vinca alkaloids–VBL (Eli Lilly Co., Indianapolis, IN); platinum compounds–CDDP (Bristol Laboratories, Princeton, NJ) and CBDCA (Bristol Laboratories, Evansville, IN); topoisomerase II inhibitors–VP-16 (Bristol Laboratories, Princeton, NJ); and taxanes–TAX (Mead Johnson, Princeton, NJ). Following incubation for 72 h at 37°C in a humidified atmosphere containing 5% CO_2, plates were washed with phosphate-buffered NaCl solution (Dulbecco's PBS, Gibco BRL, Gaithersburg, MD) and stained with 0.5% crystal violet dye in methanol. Plates were then washed three times in water and allowed to dry. Sorenson's buffer (0.025 M sodium citrate, 0.025 M citric acid in 50% ethanol) 0.1 ml was added to each well and the plates were analysed in an ELISA plate reader at 540 nm wavelength. Absorbance at this wavelength correlates closely to absolute cell number (Gillies *et al.*, 1986; Reile *et al.*, 1990; Flick and Gifford, 1984). The fraction of surviving cells relative to control were plotted against the log of drug concentration and the IC_{50} was interpolated from the resulting sigmoidal curve using a 4-parameter curve fit (SOFTmax; Molecular Devices Corporation, Menlo Park, CA).

In vivo *drug sensitivity assays*

HER-2/*neu* or control vector-infected human breast (MCF7) or ovarian (2008) carcinoma cells were injected subcutaneously at 8×10^6 ovarian cells and $0.5-1.0 \times 10^7$ breast cells/tumor in the mid-back region of 4–6 week old, female CD-1 (*nu/nu*) mice (Charles River Laboratories, Wilmington, MA). Two tumors were established in each animal. The MCF7 breast carcinoma cells were injected with an equal volume of growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) to support tumor formation. Prior to cell injection, all mice were primed with 17β-estradiol (Innovative Research of America, Sarasota, FL) applied s.c. in a biodegradable carrier binder (1.7 mg estradiol/pellet) to promote tumor cell growth. Tumor volumes were calculated as the product of length, width, and depth, and were monitored twice weekly by serial micrometer measurements by a single observer. Six to seven animals were assigned to each treatment group such that the mean starting tumor volumes were the same in each group. Very large or very small tumors were excluded from the study prior to drug treatment. Statistical tests were performed (single-factor ANOVA) to assure uniformity in starting tumor volumes between treatment groups. Chemotherapeutic drugs or isovolumetric vehicle control solution were administered by i.p. injection. The dosage of chemotherapeutic agents tested were as follows: DOX (5 mg/kg), CDDP (5 mg/kg), 5-FU (100 mg/kg), TAX (15 mg/kg, day 0,1 and 2), VP-16 (25 mg/kg, day 0, 3 and 7) and TSPA (5 mg/kg, day 0, 1 and 2). In the MCF7 xenograft model all doses and dose schedules were repeated on day 14 of the experiment. These doses and dose schedules are based on independent dose finding experiments conducted in our laboratory and are near the MTD for this specific strain and weight of female athymic mice. Doses were based on individual animal weights determined immediately prior to injection. Drug treatment was initiated on day 5 post implantation for ovarian xenografts and day 12 post implantation for breast xenografts at which time measurable growing tumor nodules had formed. Mean tumor volumes of drug-treated

relative to control-treated animals (T/C ratios) were calculated as a measure of response.

Statistical analysis

Differences between *in vitro* dose-response curves for paired (NEO *vs* HER-2) cell lines were analysed using two-factor analysis of variance (ANOVA) of data points between the IC_{20} and IC_{80}. Differences in tumor volumes following response to chemotherapy were compared using two-factor ANOVA. In addition, in the *in vivo* MCF7 breast xenograft model, differences between MCF7/NEO and MCF7/HER-2 T/C ratios were compared using non-parametric methods (Mann-Whitney U test). All statistical computations were made with Stat View SE and Super ANOVA software (Abacus Concepts, Berkeley, CA).

Note added in proof

Subsequent to the submission of this manuscript, MJ Stender, *et al.* (*Proc. Am. Soc. Clin. Oncol.*, **16,** 154a) have reported results from a clinical trial conducted by the Eastern Cooperative Oncology Group (ECOG 1193) in which patients with metastatic breast cancer were treated with doxorubicin, paclitaxel, or the combination. In this study, patients with circulating plasma c-*erb*B-2 (HER-2) extracellular domain levels $>30\,\mu$/ml ($n=61$) had statistically worse survival (median survival estimates: 17.7 months vs 30.2 months, $P=0.0008$) compared to c-*erb*B-2 negative patients; however, there was no association between quantitative c-*erb*B-2 measurements in 280 patient plasma samples and objective clinical response to chemotherapy. These clinical results are in agreement with our experimental data which indicate that HER-2/*neu* overexpression is insufficient to cause intrinsic drug resistance.

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References

Aboud-Pirak E, Hurwitz E, Pirak ME, Bellot F, Schlessinger J and Sela M. (1988). *J. Natl. Cancer Inst.*, **80,** 1605–1611.

Allred DC, Clark GM, Tandon AK, Molina R, Tormey DC, Osborne CK, Gilchrist KW, Mansour EG, Abeloff M, Eudey L and McGuire WL. (1992). *J. Clin. Oncol.*, **10,** 599–605.

Anbazhagan R, Gelber RD, Bettelheim R, Goldhirsch A and Gusterson BA. (1991). *Ann. Oncol.*, **2,** 47–53.

Arteaga CL, Winnier AR, Poirier MC, Lopez-Larraza DM, Shawver LK, Hurd SD and Stewart SJ. (1994). *Cancer Res.*, **54,** 3758–3765.

Barbareschi M, Leonardi E, Mauri FA, Serio G and Palma PD. (1992). *Am. J. Clin. Pathol.*, **98,** 408–418.

Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM and Osborne CK. (1992). *Breast Cancer Res. and Treat.*, **24,** 85–95.

Berchuck A, Rodriguez G, Kinney RB, Soper JT, Dodge RK, Clarke-Pearson DL and Bast Jr, RC. (1991). *Am. J. Obstet. Gynecol.*, **164,** 15–21.

Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B and Hynes NE. (1988). *Cancer Res.*, **48,** 1238–1242.

Berns EMJJ, Foekens JA, van Staveren IL, van Putten WLJ, de Koning HYWCM, Portengen H and Klijn JGM. (1995). *Gene*, **159,** 11–18.

Borg A, Baldetorp M, Ferno M, Killander D, Olsson H and Sigurdsson H. (1991). *Oncogene*, **6,** 137–143.

Burt RK, Garfield S, Johnson K and Thorgeirsson SS. (1988). *Carcinogenesis*, **9,** 2329–2332.

Casciari JJ, Holingshead MG, Alley MC, Mayo JG, Malspeis L, Miyauchi S, Grever MR and Weinstein JN. (1994). *J. Natl. Cancer Inst.*, **86,** 1846–1852.

Chazin VR, Kaleko M, Miller AD and Slamon DJ. (1992). *Oncogene*, **7,** 1859–1866.

Christen RD, Hom DK, Porter DC, Andrews PA, MacLeod CL, Hafstrom L and Howell SB. (1990). *J. Clin. Invest.*, **86,** 1632–1640.

Cohen BE, Egorin MJ, Kohlhepp EA, Aisner J and Gutierrez PL. (1986). *Cancer Treat. Rep.*, **70,** 859–864.

Dickstein B, Valerius EM, Wosikowski K, Saceda M, Pearson JW, Martin MB and Bates SE. (1993). *J. Cell Physiol.*, **157,** 110–118.

Dickstein BM, Wosikowski K and Bates S. (1995). *Molec. and Cell Endocrinol.*, **110,** 205–211.

D'Incalci M, Farina P, Sessa C, Mangioni C, Conter V, Masera G, Rocchetti M, Brambilla Pisoni M, Piazza E, Beer M and Cavalli F. (1982). *Cancer Chemother. Pharmacol.*, **7,** 141–145.

DiSaia PJ, Sinkovics J, Rutlege FN and Smith JP. (1972). *Am. J. Obstet. Gynecol.*, **114,** 979–989.

Endicott JA and Ling V. (1989). *Ann. Rev. Biochem.*, **58,** 137–171.

Felip E, Del Campo JM, Rubio D, Vidal MT, Colomer R and Bermejo B. (1995). *Cancer*, **75,** 2147–2152.

Flick DA and Gifford GE. (1984). *J. Immunological Methods*, **68,** 167–175.

Gillies RJ, Didier N and Denton M. (1986). *Anal. Biochem.*, **159,** 109–113.

Gormley PE, Bull JM, LeRoy AF and Cysyk R. (1979). *Clin. Pharmacol. Ther.*, **25,** 351–357.

Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, Styles J, Rudenstam C-M, Golouh R, Reed R, Martinez-Tello F, Tiltman A, Torhorst J, Grigolato P, Bettelheim R, Neville AM, Burke K, Castiglione M, Collins J, Lindtner J and Senn H-J. (1992). *J. Clin. Onc.*, **10,** 1049–1056.

Hancock MC, Langton BC, Chan T, Toy P, Monahan JJ, Mischak RP and Shawver LK. (1991). *Cancer Res.*, **51,** 4575–4580.

Harland SJ, Newell DR, Siddik ZH, Chadwick R, Hilary Calvert A and Harrup KR. (1984). *Cancer Res.*, **44,** 1693–1697.

Hetzel DJ, Wilson TO, Keeney GL, Roche PC, Cha SS and Podratz KC. (1992). *Gynecologic Oncol.*, **47,** 179–185.

Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo J, Eastman A and Howell SB. (1991). *Cancer Res.*, **51,** 5903–5909.

Kallioniema OP, Holli K, Visakorpi T, Koivula T, Helin HH and Isola JJ. (1991). *Int. J. Cancer*, **49,** 650–655.

Kerbel RS. (1995). *Invasion and Metastasis*, **14,** 50–60.

Kerbel RS, Rak J, Kobayashi H, Man MS, St Croix B and Graham CH. (1994). *Cold Spring Harbor Symposia on Quantitative Biology*, **59,** 661–672.

Klijn JGM, Berns EMJJ and Foekens JA. (1993). *Cancer Surveys Volume 18: Breast Cancer*. Sidebottom E. (ed.). Cold Spring Harbor Laboratory Press: New York, pp. 165–198.

Kobayashi H, Man S, Graham CH, Kapitain SJ, Teicher BA and Kerbel RS. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 3294–3298.

Lee AKC, Wiley B, Loda M, Dugan JM, Hamilton W, Heatley GJ, Cook L and Silverman ML. (1992). *Mod. Pathol.*, **5,** 61–67.

Liu LF. (1989). *Annu. Rev. Biochem.*, **58,** 351–375.

Lukes AS, Kohler MF, Pieper CF, Kerns BJ, Bentley R, Rodriguez GC, Soper JT, Clarke-Pearxon DL, Bast Jr, RC and Berchuck A. (1994). *Cancer*, **73,** 2380–2385.

MacMillan WE, Wolberg WH and Welling PG. (1978). *Cancer Res.*, **38,** 3479–3482.

Meyers MB, Merluzzi VJ, Spengler BA and Biedler JL. (1986). *Proc. Natl. Acad. Sci. USA*, **83,** 5521–5525.

Meyers MB, Yu P and Mendelsohn J. (1993). *Biochem. Pharmacol.*, **46,** 1841–1848.

Muss HB, Thor A, Berry DA, Kute T, Liu ET, Koerner F, Cirrincione CT, Budman DR, Wood WC, Barcos M and Henderson IC. (1994). *N. Engl. J. Med.*, **330,** 1260–1266.

Nelson RL, Dyke RW and Root MA. (1980). *Cancer Treat. Rev.*, **7** (suppl.), 17–24.

Niimi S, Nakagawa K, Yokata J, Tsuuokawa Y, Nishio K, Terashima Y, Shibuya M, Terada M and Saijo N. (1991). *Br. J. Cancer*, **63,** 237–241.

Nuti M, Zupi G, D'agnano I, Turchi V, Candiloro A and Frati L. (1991). *Anticancer Res.*, **11,** 1225–1230.

Pantel K, Schlimok Braun S, Kutter D, Lindemann F, Schaller G, Funke I, Izbicki JR and Riethmuller G. (1993). *J. Natl. Cancer Inst.*, **85,** 1419–1423.

Pauletti G, Godolphin W, Press MF and Slamon DJ. (1996). *Oncogene*, **13,** 63–72.

Peters GJ, Wets M, Keepers YPAM, Oskam R, Ark-Otte JV, Noordhuis P, Smid K and Pinedo HM. (1993). *Int. J. Cancer*, **54,** 450–455.

Pietras RJ, Fendly BM, Chazin VR, Pegram MD, Howell SB and Slamon DJ. (1994). *Oncogene*, **9,** 1829–1838.

Pietras RJ, Arboleda J, Reese DM, Wongvipat N, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX and Slamon DJ. (1995). *Oncogene*, **10,** 2435–2446.

Plowman GD, Green JM, Culouscou JM, Carlton GW, Rothwell VM and Buckley S. (1993). *Nature*, **366,** 473–475.

Poller DN, Galea M, Pearson D, Bell J, Gullick WJ, Elston CW, Blamey RW and Ellis IO. (1991). *Breast Cancer Res. and Treat.*, **20,** 3–10.

Press MF, Pike MC, Hung G, Zhou JY, Ma Y, George J, Dietz-Band J, James W, Slamon DJ, Batsakis JG and El-Naggar AK. (1994). *Cancer Res.*, **54,** 5675–5682.

Quenel N, Wafflart J, Bonichon F, de Mascarel I, Trojani M, Durand M, Avril A and Coindre JM. (1995). *Breast Cancer Res. and Treat.*, **35,** 283–291.

Querzoli P, Marchetti E, Fabris G, Marzola A, Ferretti S, Iacobelli S, Hazan R, King CR and Nenci I. (1990). *Tumori*, **76,** 461–464.

Rajkumar T and Gullick W. (1994). *Breast Cancer Res. and Treat.*, **29,** 3–9.

Reile H, Birnbock H, Bernhardt G, Sprub T and Schonenberger H. (1990). *Anal. Biochem.*, **187,** 262–267.

Robert J, Illiadis A, Hoerni B, Cano J-P, Durand M and Lagarde C. (1982). *Eur. J. Cancer Clin. Oncol.*, **18,** 739–745.

Sabbatini ARM, Basolo F, Valentini P, Mattii L, Calvo S, Fiore L, Ciardiello F and Petrini M. (1994). *Int. J. Cancer*, **59,** 208–211.

Saffari B, Jones LA, el-Naggar A, Felix JC, George J and Press MF. (1995). *Cancer Res.*, **55,** 5693–5698.

Scanlon KJ, Kashani-Sabet M, Miyachi H, Sowers LC and Rossi J. (1989). *Anticancer Res.*, **9,** 1301–1312.

Semba K, Kamata N, Toyoshima K and Yamamoto T. (1985). *Proc. Natl. Acad. Sci. USA*, **82,** 6497–6501.

Seshadri R, Firgaira FA, Horsfall DJ, McCaul K, Setlur V and Kitchen P. (1993). *J. Clin. Oncol.*, **11,** 1936–1942.

Sklar MD and Prochownik EV. (1991). *Cancer Res.*, **51,** 2118–2123.

Slamon DJ, Clark GM, Wong SJ, Levin WJ, Ullrich A and McGuire WL. (1987). *Science*, **235,** 177–182.

Sliwkowski MX, Schaefer G, Akita RW, Lofren JA, Fitzpatrick VD, Nuijens A, Fendley BM, Cerione RA, Vandlen RL and Carraway III K. (1994). *J. Biol. Chem.*, **269,** 14661–14665.

Smith K, Houlbrook S, Greenall M, Carmichael J and Harris AL. (1993). *Oncogene*, **8,** 933–938.

Stal O, Sullivan S, Sun XF, Wingren S and Nordenskjold B. (1994). *Cytometry*, **16,** 160–168.

Tsai C-M, Chang K-T, Perng R-P, Mitsudomi T, Chen M-H, Kadoyama C and Gazdar AF. (1993). *J. Natl. Cancer Inst.*, **85,** 897–901.

Tsai C-M, Yu D, Chang K-T, Wu L-H, Perng R-P, Ibrahim NK and Hung M-C. (1995). *J. Natl. Cancer Inst.*, **87,** 682–684.

Wiernik PH, Schwartz EL, Strauman JJ, Dutcher JP, Lipton RB and Paietta E. (1987). *Cancer Res.*, **47,** 2486–2493.

Wright C, Cairns J, Cantwell BJ, Cattan AR, Hall AG, Harris AL and Horne CHW. (1992). *Br. J. Cancer*, **65,** 271–274.

Yonemura Y, Ninomiya I, Yamaguchi A, Fushida S, Kimura H, Ohoyama S, Miyazaki I, Endou Y, Tanaka M and Sasaki T. (1991). *Cancer Res.*, **51,** 1034–1038.

Yu D, Liu B, Tan M, Li J, Wang S-S and Hung M-C. (1996). *Oncogene*, **13,** 1359–1365.

Oncogene (1998) 17, 2235–2249

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Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs

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HER-2 oncogene encodes a transmembrane growth factor receptor that is overexpressed in 25–30% of patients with primary breast and ovarian cancer. A murine monoclonal antibody, 4D5, to the extracellular domain of HER-2 receptor elicits cytostatic growth inhibition of tumor cells overexpressing HER-2 protein, but clinical use of this antibody is limited by genesis of human anti-mouse antibodies. To avoid this problem, a recombinant humanized 4D5 monoclonal antibody (rhuMAb HER-2) was developed and tested using a human tumor xenograft model. Human breast and ovarian cancer cells which overexpress HER-2 were inhibited *in vivo* by the rhuMAb HER-2 antibody. Tumor growth relative to control was reduced at all doses of antibody tested, and the magnitude of growth inhibition was directly related to dose of rhuMAb HER-2. Tumor growth resumed on termination of antibody therapy, indicating a cytostatic effect. To elicit a cytotoxic response, human breast tumor xenografts were treated with a combination of antibody and antitumor drugs, cisplatin or doxorubicin. The combination of antibody with either cisplatin or doxorubicin resulted in significantly greater growth inhibition, with the cisplatin combination demonstrating a greater response. In addition, therapy with cisplatin and antireceptor antibody elicited complete tumor remissions after 2–3 cycles of therapy. The schedule of administration of antireceptor antibody and cisplatin was critical for occurrence of antibody-induced potentiation in cisplatin cytotoxicity. Enhanced killing of tumor cells was found only if antibody and drug were given in close temporal proximity. Since interference with DNA repair pathways may contribute to this receptor-enhanced chemosensitivity, repair of cisplatin-damaged reporter DNA (pCMV-β) was determined in human breast cells. As in studies of antibody-enhanced cisplatin cytotoxicity *in vivo*, treatment with rhuMAb HER-2 blocked the repair of cisplatin-damaged DNA only if the antibody was administered in close temporal proximity to transfection of the drug-exposed reporter DNA. An alternative measure of DNA repair, unscheduled DNA synthesis, was also assessed. Treatment with either cisplatin or doxorubicin led to an increase in unscheduled DNA synthesis that was reduced by combined therapy with antireceptor antibody specific to HER-2-overexpressing breast cancer cells. Using a direct measure of DNA repair, therapy of HER-2-overexpressing cells with rhuMAb HER-2 also blocked the removal of cisplatin-induced DNA adducts. Expression of p21/WAF1, an important mediator of DNA repair, was disrupted in breast cancer cells with HER-2 overexpression, but not in control cells, after treatment with HER-2 antibody, thus suggesting cross-communication between the HER-2 signaling and DNA repair pathways. These data demonstrate an *in vivo* antiproliferative effect of rhuMAb HER-2 on tumors that overexpress HER-2 receptor and a therapeutic advantage in the administration of the antireceptor antibody in combination with chemotherapeutic agents.

Keywords: breast cancer; ovarian cancer; monoclonal antibody; HER-2/neu; cisplatin; doxorubicin

Introduction

Breast cancer is a leading cause of cancer-related death in women, with ultimate treatment failure often related to resistance to conventional drug therapy (Harris *et al*., 1992). Screening studies of human breast cancer tissue for genetic alterations revealed amplification and/or overexpression of HER-2 (c-*erb*B-2/neu) proto-oncogene in 25–30% of these cancers (Slamon *et al*., 1987, 1989a; Harris *et al*., 1992). This molecular alteration correlates with a poor prognosis in that patients whose tumors contain the alteration have a shorter disease-free survival as well as a shorter overall survival (Slamon *et al*., 1987, 1989a; Lemoine *et al*., 1990; Press *et al*., 1993; Seshadri *et al*., 1993). Moreover, results of recent clinical trials suggest that improvement in the outcome of patients with HER-2-overexpressing breast cancer may require treatment with significantly higher doses of combination chemotherapy including anthracyclines and alkylating agents (Muss *et al*., 1994).

The HER-2 proto-oncogene encodes a 185 000 kd transmembrane receptor tyrosine kinase with homology to epidermal growth factor receptor (Coussens *et al*., 1985; Semba *et al*., 1985). This receptor has oncogenic potential which may be mediated through multiple genetic mechanisms including point mutations in the transmembrane domain (Bargmann *et al*., 1986), truncation of the extracellular domain or overexpression of the non-mutated proto-oncogene (DiFiore *et al*., 1987; Hudziak *et al*., 1987; Yarden and Ullrich, 1988; Aaronson, 1991). To date, no similar point mutations or truncations have been found in the HER-2 gene product in human cancers (Slamon *et al*., 1987; 1989a,b; Aaronson, 1991; Lofts and Gullick, 1992). Rather, the alteration occurring in human malignant cells is overexpression of a normal gene product which is almost always but not uniformly due to gene

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amplification (Slamon *et al.*, 1989a,b; Lemoine *et al.*, 1990; Pauletti *et al.*, 1996). In addition, overexpression of structurally-unaltered HER-2 gene leads to neoplastic transformation of both NIH3T3 cells (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987) and immortalized, but non-transformed, human breast cells (Pierce *et al.*, 1991), indicating that this alteration may play a pathogenic role in promoting tumorigenicity of non-malignant cells. Collectively, such data indicate that amplification and/or overexpression of the HER-2 gene in human breast cells has a significant effect on their biologic behavior and support the concept that this alteration plays a pathogenic role in increasing growth and tumorigenicity of human breast cancer cells.

Monoclonal antibodies against the extracellular domain of HER-2 membrane receptor can suppress tumorigenesis by HER-2-transformed NIH3T3 or NR6 cells (Drebin *et al.*, 1988; Chazin *et al.*, 1992) and specifically inhibit the growth of human breast carcinoma cells overexpressing the HER-2 gene product (Hudziak *et al.*, 1989). One murine monoclonal antibody, 4D5, has proven particularly effective in inhibiting growth of human tumor cells with HER-2 overexpression (Hudziak *et al.*, 1989; Fendly *et al.*, 1990). However, available data indicate that effects of 4D5 antibody are cytostatic, not cytocidal. A second difficulty with the antibody is that it is a mouse product and as such can elicit a human anti-mouse antibody response in patients receiving it. To circumvent this problem, a humanized version of 4D5 was developed (Carter *et al.*, 1992). This engineered antibody contains only the antigen binding loops from murine antibody 4D5 and includes human variable region framework residues plus human IgG1 constant domains (Carter *et al.*, 1992). Prior pharmacokinetic studies using murine monoclonal antibody 4D5 and rhuMAb HER-2 (Maneval *et al.*, 1991; DeSantes *et al.*, 1992) have been presented. These data show that *in vivo* serum clearance and permanence times are similar for humanized and native murine monoclonal antibodies. The efficacy of recombinant humanized monoclonal antibody to HER-2 receptor (rhuMAb HER-2) *in vitro* on human breast cells with overexpression of HER-2 receptor has also been demonstrated (Carter *et al.*, 1992), but the effect of this preparation *in vivo* in preclinical animal studies remains to be established.

Independent studies show that ligands or antibodies to growth factor receptors can potentiate the cytotoxicity of chemotherapeutic drugs (Aboud-Pirak *et al.*, 1988; Jensen and Linn, 1988; Christen *et al.*, 1991; Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1994; Arteaga *et al.*, 1994; Dixit *et al.*, 1997; Mendelsohn and Fan, 1997). Monoclonal antibodies to EGF receptor elicited an additive antitumor effect when given in combination with the anthracycline drug, doxorubicin (Aboud-Pirak *et al.*, 1989). A poorly understood but probable synergistic effect between monoclonal antibodies to EGF receptor and the chemotherapy drug, cisplatin, has also been reported (Aboud-Pirak *et al.*, 1988). The combined treatment resulted in a dramatic reduction in the number and size of epidermoid cancers grown as xenografts in athymic mice. Antibodies to HER-2 receptor have likewise been found to promote cell killing by cisplatin in tumors with overexpression of the HER-2 membrane receptor (Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1994), and this effect has been shown to be a true synergistic interaction in both breast and ovarian cancer cells (Pietras *et al.*, 1994). Similarly, binding of certain growth factors to their cognate receptors has been reported to modulate cellular sensitivity to drugs. Incubation of human tumor cells with EGF has been found to increase sensitivity of these cells to the cytotoxic effects of cisplatin (Christen *et al.*, 1991). A biologic basis for these growth factor receptor-dependent changes in cellular sensitivity to DNA-interacting agents may be related to DNA repair mechanisms. Treatment of human neuroblastoma cells with NGF slows the removal of DNA adducts caused by the DNA-damaging drug, benzo(a)pyrene (Jensen and Linn, 1988). Signal generated by activation of EGF receptor may also alter the rate of DNA repair in affected cells (Christen *et al.*, 1991). Work from our laboratory shows that anti-HER-2 receptor antibody-induced blockade of cisplatin-DNA adduct repair in cells with HER-2 overexpression leads to a two log increase in cytotoxicity of the drug (Pietras *et al.*, 1994). The specific molecular pathway for suppression of DNA repair triggered by ligand (or antireceptor antibody) interactions remains undefined. Cell responses to DNA damage are regulated, in part, by growth factor signaling pathways (Canman *et al.*, 1995; Yen *et al.*, 1997). Recent reports show p53-independent activation of p21/WAF1 by mitogen-activated protein kinase (MAP kinase) signaling, and withdrawal of growth factors *in vitro* has been associated with down-regulation of p21/WAF1 expression and with enhanced cell killing in response to DNA damage (Canman *et al.*, 1995; Liu *et al.*, 1996). Since maintenance of the integrity of DNA by repair is essential to cell survival, blockade of DNA repair triggered by peptide ligand or antireceptor antibody interactions could have application in cancer therapy.

The objectives of this study are to further evaluate the possibility of therapeutically exploiting these types of interactions to treat human cancer cells which overexpress the HER-2 receptor. The data presented demonstrate an *in vivo* cytostatic effect of rhuMAb-HER-2 in both breast and ovarian cancer cells with HER-2 overexpression. On the basis of independent work showing synergistic interaction between 4D5 antireceptor antibody and the DNA-damaging drug, cisplatin, resulting in enhanced cytotoxicity in tumors, the therapeutic advantage of rhuMAb HER-2 given in combination with cisplatin was tested with human breast tumor xenografts in athymic mice. In addition, comparison of rhuMAbHER-2 interaction with the DNA-intercalating drug, doxorubicin, was conducted. These studies reveal that the humanized antireceptor antibody enhances breast cancer cell killing in combination with some chemotherapeutic agents, with optimal antitumor effects occurring in combination with cisplatin. The HER-2 receptor-enhanced sensitivity to cisplatin occurred only if the two agents were administered in close temporal proximity, suggesting a critical biologic timeframe for promoting this phenomenon. These results provide a tentative schedule for testing and exploiting this novel therapeutic strategy in the clinic.

Results

Effect of recombinant humanized monoclonal antibody to HER-2 (rhuMAb HER-2) on growth of human breast and ovarian cells in athymic mice

Introduction of full-length human HER-2 cDNA into human breast cancer cells, MCF-7, results in 2–5 copies of the gene per cell as compared to 5–8 copies of the gene in SKBR3 cells, a non-engineered, naturally-amplified cell line from patient material which expresses levels of the gene at the upper limit of that seen in human malignancies in nature (Kraus *et al.*, 1987). A similar level of amplification is observed after transfection of CAOV3 cells with HER-2 retroviral vector. Levels of HER-2 overexpression as assessed by Western blot analyses demonstrate expression levels at or slightly below those seen in the naturally HER-2-amplified, overexpressing SKBR3 cells. Such overexpression of the gene in murine cells has profound biologic effects, including significant increments in DNA synthesis, cell growth, cloning efficiency in soft agar, and in tumor formation in nude mice as reported previously (Chazin *et al.*, 1992; Pietras *et al.*, 1994, 1995). Although MCF-7 parent and MCF-7 control cells form tumors in nude mice with estrogen treatment as reported before (Soule and McGrath, 1980), overexpression of the HER-2 gene in human breast cancer cells (MCF-7/HER-2) leads to formation of tumors in nude mice at 10-times the size of those formed by MCF-7 parent or MCF-7 /CON cells after 28 days ($P<0.001$; see Figure 1).

To determine if the rhuMAb HER-2 monoclonal antibody which is directed against the extracellular domain of the human gene had any effect on human cancer cells overexpressing the HER-2 gene, studies were performed using this antibody to treat nude mice implanted with the engineered human breast and ovarian cancer cells. Overexpressing MCF-7 human breast or overexpressing CaOV3 human ovarian cancer cells were injected subcutaneously at a dose of $3.5-5.0\times10^7$ cells/animal in the mid-back region of 3-month-old female Swiss nude mice which had been primed for 7 days with estradiol-17β. Following injection of cells, a period of 7 days elapsed to allow formation of tumor nodules. Animals were then randomized into six uniform groups based on animal weight and tumor volume at the start of the experiment. Monoclonal antibody and control solution were administered by intraperitoneal injection. RhuMAb HER-2 was tested at total doses of 3, 10, 30 and 100 mg/kg and compared to the known *in vivo* inhibitory effects of the murine 4D5 antibody. Control injections included huIgG1, total dose 100 mg/kg, and murine MAb 4D5, total dose 25 mg/kg. As indicated in Materials and methods, our choices for dose and schedule of therapy were based on results of prior pharmacokinetic studies using murine monoclonal antibody 4D5 and rhuMAb HER-2 (Maneval *et al.*, 1991; DeSantes *et al.*, 1992). Test agents were administered in three divided doses on days 1, 5 and 9. Tumor nodules were monitored two times per week by serial micrometer measurements by a single observer. Tumor size in treated animals was followed to day 21.

Results of studies with MCF-7 /HER-2 cells are shown in Figure 2. The effect of various doses of rhuMAb HER-2 (Groups C–F) on tumor volume was compared to that of control human IgG1 (Group A) and rhuMAb 4D5 (Group B). Marked inhibition of tumor growth relative to control was seen at all

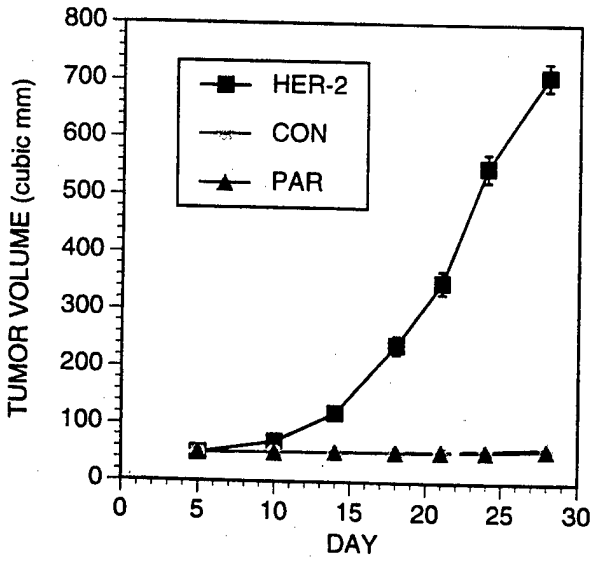

Figure 1 Growth of MCF-7 cells with or without HER-2 gene overexpression as xenografts in nude mice. MCF-7 parental cells (MCF–PAR) were bioengineered with CON (normal-copy HER-2) or HER-2 (multi-copy HER-2) retroviral expression vectors as described in Materials and methods. Cells were inoculated subcutaneously in athymic mice which had been primed for 7 days with estradiol-17β. Tumor nodules were then monitored to day 28

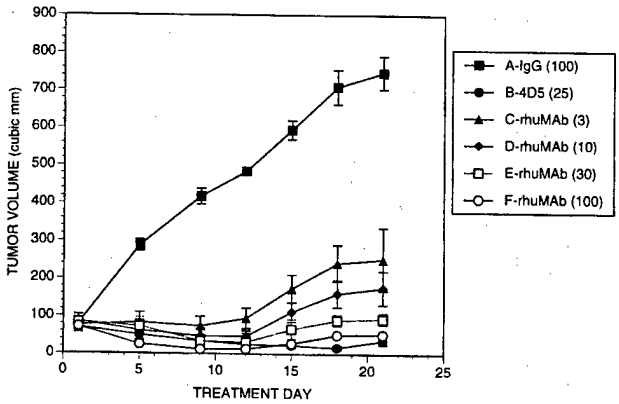

Figure 2 Antitumor efficacy of various doses of rhuMAb HER-2 on human MCF-7 breast tumor xenografts in athymic mice. MCF-7 cells were engineered for overexpression of HER-2 receptor as described in Materials and methods. After 7 days, treatments were instituted with human IgG1 at 100 mg/kg (Group A); murine monoclonal antibody 4D5 at 25 mg/kg (Group B); or rhuMAb HER-2 at 3 mg/kg (Group C), 10 mg/kg (Group D), 30 mg/kg (Group E) or 100 mg/kg (Group F). Mean tumor size in each rhuMAb HER-2 group was compared to that in human IgG1- or murine 4D5-treated groups. Marked inhibition of mean day 21 tumor growth relative to control IgG1 was observed at all doses of rhuMAb HER-2 tested ($P<0.01$). Animal weights on days 1 and 21 were not significantly different (data not shown)

doses of rhuMAb HER-2 tested ($P<0.01$). Analyses of mean tumor volumes at day 21 indicate that the antitumor effect of rhuMAb HER-2 is dose-dependent ($P<0.01$). The rhuMAb HER-2 at a dose of 100 mg/kg had an effect comparable to murine 4D5 antibody at a dose of 25 mg/kg. It is notable that rhuMAb HER-2, even at the lowest dose tested (3 mg/kg), effectively suppressed tumor growth during the period of active treatment (i.e., day 1 through day 9). In independent control experiments, we also tested the effect of rhuMAb HER-2 at a dose of 30 mg/kg in estrogen-supplemented nude mice innoculated with MCF-7 / CON tumors at 50--100 mm^3 in size. After 21 days of therapy as above, no significant antitumor effect of the antibody was found in tumors induced by cells with a single-copy of the gene which express normal levels of the HER-2 receptor (data not shown).

A parallel study of rhuMAb HER-2 effects in CAOV3/HER-2 human ovarian cancer cells is shown in Figure 3. As with the breast cancer cells, the antitumor effect of several doses of rhuMAb HER-2 (Groups C–F) on human ovarian cancer cells was compared to that of control human IgG1 (Group A) and murine 4D5 (Group B) treatment over a 21 day period. Inhibition of tumor growth at day 21 relative to control IgG1 was observed at all doses of rhuMAb HER-2 tested. The degree of inhibition reached statistical significance at the highest dose of rhuMAb HER-2 where a tenfold decrease in tumor size compared to control was found ($P<0.001$). These data demonstrate that the tumor suppressive activity of the rhuMAb HER-2 monoclonal antibody is not restricted by cell or epithelial tissue type.

Effect of combined therapy with rhuMAb HER-2 and cisplatin in athymic mice with human breast tumor xenografts

In view of recent reports indicating that murine anti-HER-2 receptor antibodies have synergistic antitumor effects with cisplatin (Christen *et al.*, 1991; Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1994), experiments were conducted to evaluate potential enhanced effects of rhuMAb HER-2 when combined with the chemotherapeutic drug, cisplatin, on the growth of HER-2-overexpressing human breast cancer cells. The MCF-7 /HER-2 cells were cultivated in estrogen-primed female nude mice for 7 days and then randomized to seven treatment groups. The study design included mice treated with: human IgG1 control at 3 mg/kg (Group A); cisplatin at 0.25 mg/kg and IgG1 at 3 mg/kg (Group B); cisplatin at 0.75 mg/kg and IgG1 at 3 mg/kg (Group C); rhuMAb HER-2 at 1 mg/kg (Group D) and at 3 mg/kg (Group E); rhuMAb HER-2 at 1 mg/kg with cisplatin at 0.25 mg/kg (Group F); rhuMAb HER-2 at 1 mg/kg with cisplatin at 0.75 mg/kg (Group G); rhuMAb HER-2 at 3 mg/kg with cisplatin at 0.25 mg/kg (Group H); and rhuMAb HER-2 at 3 mg/kg with cisplatin at 0.75 mg/kg (Group I). The total doses of antibody indicated above were administered as three divided doses on days 1, 5 and 9. Those groups treated with cisplatin received a single injection of the drug 18 h after administration of the antibody. All agents were given as intraperitoneal injections. Tumor nodules were monitored up to day 21.

Figure 4 shows the magnitude and time course of the effect of various doses of rhuMAb HER-2 with or

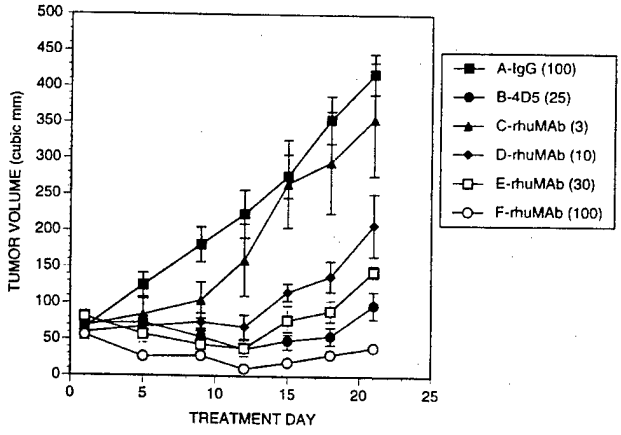

Figure 3 Antitumor efficacy of various doses of rhuMAb HER-2 on human CAOV3 ovarian tumor xenografts in athymic mice. CAOV3 cells were engineered for overexpression of HER-2 receptor as described in Materials and methods. After 7 days, treatments were instituted with human IgG1 at 100 mg/kg (Group A); murine monoclonal antibody 4D5 at 25 mg/kg (Group B); or rhuMAb HER-2 at 3 mg/kg (Group C), 10 mg/kg (Group D), 30 mg/kg (Group E) or 100 mg/kg (Group F). The antitumor effect of the several doses of rhuMAb HER-2 was compared to that of control human IgG1 and 4D5 treatments over 21 days. Inhibition of tumor growth at day 21 relative to control IgG1 was observed at all doses of rhuMAb HER-2 tested, but only reached statistical significance at the highest dose of rhuMAb HER-2 where a tenfold decrease in tumor size compared to control was found ($P<0.001$). Animal weights on days 1 and 21 were not significantly different (data not shown)

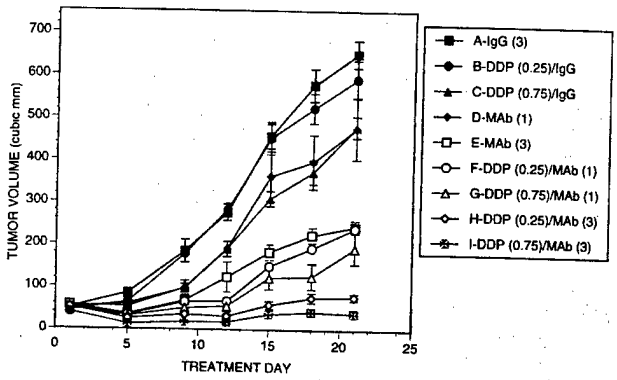

Figure 4 Enhanced antitumor effects of the chemotherapeutic drug, cisplatin, when combined with rhuMAb HER-2. HER-2-overexpressing MCF-7 breast cancer cells were cultivated in estrogen-primed female nude mice for 7 days and then randomized to seven treatment groups. The study design included mice treated with: human IgG1 control at 3 mg/kg (Group A); cisplatin at 0.25 mg/kg and IgG1 at 3 mg/kg (Group B); cisplatin at 0.75 mg/kg and IgG1 at 3 mg/kg (Group C); rhuMAb HER-2 at 1 mg/kg (Group D) and at 3 mg/kg (Group E); rhuMAb HER2 at 1 mg/kg with cisplatin at 0.25 mg/kg (Group F); and rhuMAb HER-2 at 1 mg/kg with cisplatin at 0.75 mg/kg (Group G); rhuMAb HER-2 at 3 mg/kg with cisplatin at 0.25 mg/kg (Group H); and rhuMAb HER-2 at 3 mg/kg with cisplatin at 0.75 mg/kg (Group I). Total doses of antibody above were administered as three divided doses on days 1, 5 and 9. Groups treated with cisplatin received a single injection of the drug 18 h after antibody. All agents were given as rapid intraperitoneal injections. Tumor nodules were monitored until day 21

without cisplatin on tumor volume compared to control groups. Results at days 18 and 21 were comparable and are detailed here. Of mice receiving either rhuMAb HER-2 at low dose (Group D) or cisplatin with control IgG (Groups B,C), mean inhibition of tumor growth compared to control (Group A) was measurable but minimal ($P>0.05$) and only attained statistical significance in animals receiving 3 mg/kg of rhuMAb HER-2 ($P<0.01$). In contrast, animals that received both rhuMAb HER-2 and a single injection of cisplatin displayed a marked reduction of 2–16-fold in mean 21-day tumor volumes relative to control ($P<0.01$). Moreover, average tumor sizes in animals injected with both rhuMAb HER-2 and cisplatin (i.e., Groups G–I) were, with the exception of Group F, significantly less than when comparable doses of either agent were given separately ($P<0.05$). These data indicate an enhanced effect of cisplatin when administered with rhuMAb HER-2 and support the clinical application of these agents in combination.

Effect of order of administration of rhuMAb HER-2 and cisplatin on growth of human breast tumor xenografts in athymic mice

To evaluate the potential influence of schedule of administration of rhuMAb HER-2 when combined with cisplatin on the growth of HER-2-overexpressing MCF-7 cells, the cells were cultivated in estrogen-primed female athymic mice for 14 days and then randomized to one of 18 treatment groups. The study design is outlined in Table 1. Doses of antibody were administered as indicated in the Table at various times before or after cisplatin. All agents were given as intraperitoneal injections. Tumor nodules were monitored to day 21.

The effect of rhuMAb HER-2 given at various times before or after cisplatin on breast tumor volume compared to control groups is demonstrated in Figure 5. In these experiments, rhuMAb HER-2 was given at 3 mg/kg, and cisplatin was used at a dose of 0.5 mg/kg. In Figure 5a, rhuMAb HER-2 is injected on days 1, 2, 3 or 5, with the antitumor effect compared to IgG control given at day 1. All treatments with antibody alone elicited a significant growth suppression as compared to control ($P<0.05$). In Figure 5b, cisplatin at 0.5 mg/kg is administered with IgG at days 1, 2, 3 or 5. Therapy with cisplatin on the several days tested also blocked tumor formation in athymic mice as compared to the IgG control group ($P<0.05$).

Several different combination treatments with antibody and drug are presented in Figure 5c and d. As shown in Figure 5c, rhuMAb HER-2 is given on day 1, with cisplatin administration varying from day 1 through day 5. Each of these treatment protocols promoted significant growth suppression as compared to the IgG control group ($P<0.01$). With the exception of Groups 13 and 14 (c.f. Table 1) in which cisplatin followed antibody by 3 to 5 days, the groups exhibited significantly more tumor growth inhibition than mice treated with cisplatin alone ($P<0.05$). The final set of treatment protocols is shown in Figure 5d which presents data from mice given cisplatin on day 1, with rhuMAb HER-2 administration varying from day 1 through day 5 (cf. Table 1). All treatments with cisplatin followed by antibody showed a significant antitumor effect compared to IgG controls ($P<0.05$); however, cisplatin followed by rhuMAb HER-2 at days 2 to 5 did not elicit greater tumor growth suppression than antibody given alone on corresponding days ($P>0.25$). Moreover, administration of rhuMAb HER-2 at 1–4 days after cisplatin (Groups 16–18) showed less antitumor efficacy than those regimens in which antireceptor antibody preceded cisplatin (Groups 10–12; $P<0.05$). These data demonstrate that the order of antibody/cisplatin administration is critical and clearly affects the magnitude of observed antitumor responses in HER-2-overexpressing human breast cancer xenografts.

Table 1 Effect of order of administration of rhuMAb HER-2 and cisplatin on growth of HER-2-overexpressing human breast tumor xenografts in athymic mice

Group[a]	*Test agents*[b]	*Injection time*[c]	*Dose*[d]
1	Control IgG[e]	Day 14	3
2	rhuMAb HER-2	Day 14	3
3	rhuMAb HER-2	Day 15	3
4	rhuMAb HER-2	Day 17	3
5	rhuMAb HER-2	Day 19	3
6	Control IgG/Cisplatin[f]	Day 14	0.5
7	Control IgG/Cisplatin	Day 15	0.5
8	Control IgG/Cisplatin	Day 17	0.5
9	Control IgG/Cisplatin	Day 19	0.5
10	rhuMAb HER-2/Cisplatin[g]	Day 14/Day 14	3/0.5
11	rhuMAb HER-2/Cisplatin	Day 14/Day 14+8 h	3/0.5
12	rhuMAb HER-2/Cisplatin	Day 14/Day 15	3/0.5
13	rhuMAb HER-2/Cisplatin	Day 14/Day 17	3/0.5
14	rhuMAb HER-2/Cisplatin	Day 14/Day 19	3/0.5
15	Cisplatin/rhuMAb HER-2	Day 14/Day 14+8 h	0.5/3
16	Cisplatin/rhuMAb HER-2	Day 14/Day 15	0.5/3
17	Cisplatin/rhuMAb HER-2	Day 14/Day 17	0.5/3
18	Cisplatin/rhuMAb HER-2	Day 14/Day 19	0.5/3

[a]Five mice per group. Cells were grown in estrogen-primed female athymic mice for 14 d and randomized to 18 treatment groups. [b]Order of injections are shown when both test agents are given. Doses of antibody were administered as indicated at various times before or after cisplatin. [c]Time of dosing defined here from date of tumour innoculation; for example, day 14 = day 1 for injection of test agents. [d]Agent given as mg/kg mouse body weight, with all agents given as intraperitoneal injections. [e]Nonspecific human IgG1. [f]Control IgG dose (3 mg/kg) precedes cisplatin by 1 min. [g]RhuMAb HER-2 dose precedes cisplatin by 1 min

Effect of cyclic therapy with cisplatin and rhuMAb HER-2 on human breast tumor growth in nude mice

To evaluate the cytotoxic efficacy of repeated therapy with rhuMAb HER-2 in combination with cisplatin on the growth of HER-2-overexpressing MCF-7 cells, cells were cultivated in estrogen-primed female athymic mice for 14 days and then randomized to four groups for three cycles of therapy. Treatment groups included human IgG1 control at 30 mg/kg (CON), cisplatin at 5 mg/kg with human IgG1 (DDP), rhuMAb HER-2 at 30 mg/kg (rhuMAb) or combined cisplatin / rhuMAb (rhuMAb/DDP) therapy. Doses of rhuMAb HER-2 antibody or IgG1 control were administered in divided doses on days 1, 5 and 9, repeated on days 21, 25 and 29 and once again on days 42, 46 and 50. The groups treated with cisplatin received a single injection of the drug immediately after administration of the antibody or IgG1. All agents were given as intraperitoneal injections, and tumor nodules were monitored until day 64.

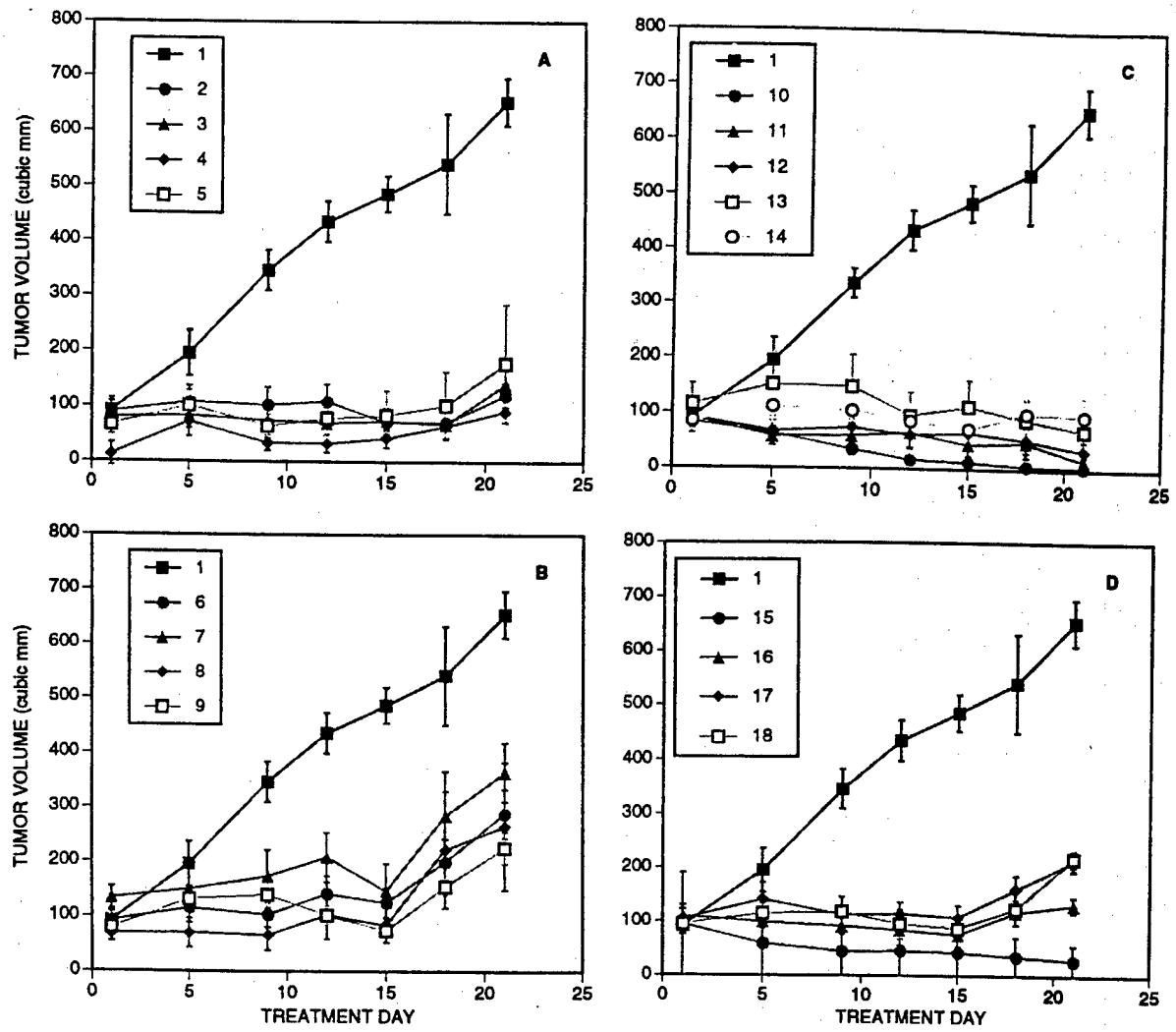

Figure 5 Influence of order of administration of rhuMAb HER-2 and cisplatin on the growth of human MCF-7/HER-2 breast tumor xenografts in nude mice over 21 days. (**a**) Mean tumor volumes of mice treated with human IgG1 on day 1 as compared to therapy with rhuMAb HER-2 on days 1, 2, 3 or 5 (c.f. Groups 1–5 in Table 1.). (**b**) Mean tumor volumes of mice treated with human IgG1 on day 1 as compared to therapy with cisplatin on days 1, 2, 3 or 5 (c.f. Groups 1 and 6–9 in Table 1.). (**c**) Mean tumor volumes of mice treated with human IgG1 on day 1 as compared to therapy with rhuMAb HER-2 on day 1 followed by cisplatin on days 1–5 (c.f. Groups 1 and 10–14 in Table 1.). (**d**) Mean tumor volumes of mice treated with human IgG1 on day 1 as compared to therapy with cisplatin on day 1 followed by rhuMAb HER-2 on days 1–5 (c.f. Groups 1 and 15–18 in Table 1.)

Figure 6 shows the effect of repeated doses of rhuMAb HER-2 with or without cisplatin on tumor volume compared to control groups. In mice receiving cisplatin with control IgG (DDP), mean tumor volumes compared to control (CON) were reduced over the 9-week treatment period ($P<0.001$), but no complete tumor remissions were observed. Tumors exposed to rhuMAb HER-2 alone (rhuMAb) also showed reduced growth ($P<0.001$) as compared to controls (CON), but, again, no complete tumor remissions were obtained. In contrast, combined drug/antibody therapy produced a marked reduction in tumor volumes compared to control values ($P<0.001$), and five of six animals receiving both rhuMAb HER-2 and cisplatin (rhuMAb/DDP) had complete tumor remissions after 2–3 cycles of therapy, with a partial remission occurring in the remaining animal. Effects of combined drug-antibody therapy were significantly different from those found with antibody or cisplatin treatment alone ($P<0.001$). These data show markedly increased cytotoxicity of cisplatin when administered with rhuMAb HER-2 and support the potential clinical utility of these agents in combination.

Effect of cyclic therapy with doxorubicin and rhuMAb HER-2 on human breast tumor growth in nude mice

Prior work has shown some therapeutic advantage in the treatment of human tumors with anti-EGF receptor antibodies and doxorubicin (Aboud-Pirak *et al.*, 1989), a drug commonly used in the treatment of breast cancer. Although anthracyclines are not generally considered to be DNA-damaging agents, recent work suggests these agents may elicit some indirect covalent modifications of DNA in mammary tissue (Purewal and Liehr, 1993). To evaluate the efficacy of therapy with rhuMAb HER-2 in combina-

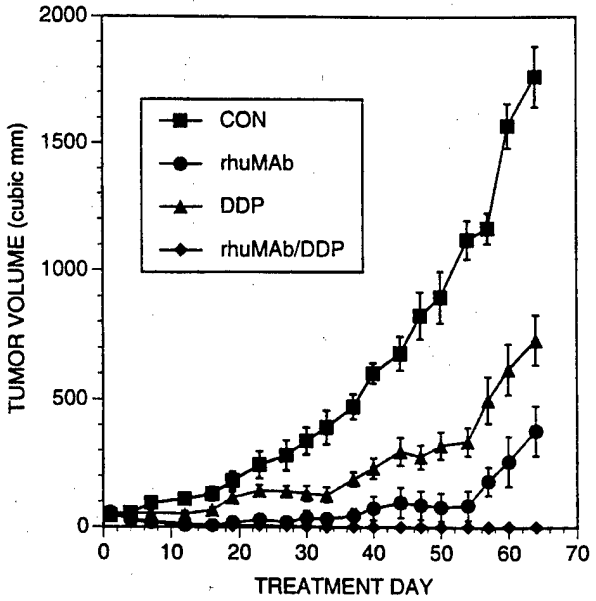

Figure 6 Effect of cyclic therapy with cisplatin and rhuMAb HER-2 on growth of MCF-7/HER-2 breast tumor xenografts in nude mice over 64 days. Cells were cultivated in estrogen-primed female nude mice for 7 days and then randomized to four treatment groups. The study design included the following groups: human IgG1 control at 30 mg/kg given in divided doses at days 1, 4 and 9, and then repeated on days 21, 25 and 29 and finally on days 42, 46 and 50 (CON); IgG1 and cisplatin at 5 mg/kg given as a single dose on days 1, 21 and 42 (DDP); rhuMAb HER-2 at 30 mg/kg given in divided doses at days 1, 4 and 9, and then repeated on days 21, 25 and 29 and finally on days 42, 46 and 50 (rhuMAb); and cisplatin combined with rhuMAb HER-2 (rhuMAb/DDP). Those groups treated with cisplatin received a single injection of the drug immediately after administration of antibody or IgG1. All agents were given as rapid intraperitoneal injections. Tumor nodules were monitored until day 64

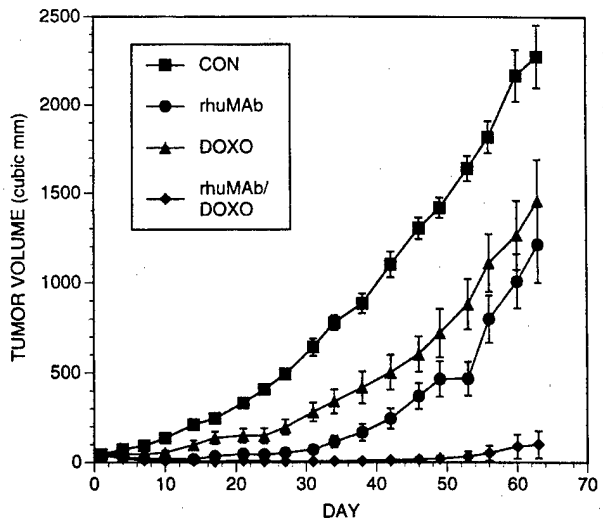

Figure 7 Effect of cyclic therapy with doxorubicin and rhuMAb HER-2 on growth of MCF-7/HER-2 breast tumor xenografts in nude mice over 64 days. Cells were cultivated in estrogen-primed female nude mice for 7 days and then randomized to four treatment groups. The study design included the following groups: human IgG1 control at 30 mg/kg given in divided doses at days 1, 4 and 9 and then repeated on days 21, 25 and 29 and finally on days 42, 46 and 50 (CON); IgG1 and doxorubicin at 5 mg/kg given as a single dose on days 1, 21 and 42 (DOXO); rhuMAb HER-2 at 30 mg/kg given in divided doses at days 1, 4 and 9 and then repeated on days 21, 25 and 29 and finally on days 42, 46 and 50 (rhuMAb); and doxorubicin combined with rhuMAb HER-2 (rhuMAb/DOXO). Those groups treated with doxorubicin received a single injection of the drug immediately after administration of antibody or IgG1. All agents were given as rapid intraperitoneal injections. Tumor nodules were monitored until day 64

tion with doxorubicin on the growth of HER-2-overexpressing MCF-7 cells, cells were cultivated in estrogen-primed female athymic mice for 14 days and then randomized to four groups for three cycles of therapy as above. Treatment groups included human IgG1 control at 30 mg/kg (CON) doxorubicin at 5 mg/kg with human IgG1 (DOXO), rhuMAb HER-2 at 30 mg/kg (rhuMAb) or combined doxorubicin/ rhuMAb (rhuMAb/DOXO) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1, 5 and 9 and then repeated on days 21, 25 and 29 and finally on days 42, 46 and 50. Those groups treated with doxorubicin received a single injection of the drug immediately after administration of the rhuMAb HER-2 antibody or control IgG1. All agents were given as intraperitoneal injections. Tumor nodules were monitored to day 64.

Figure 7 shows the effect of repeated doses of rhuMAb HER-2 with or without doxorubicin on tumor volume as compared to control groups. Mice receiving doxorubicin with control IgG (DOXO) had mean tumor volumes compared to control (CON) which were significantly reduced over the 9-week treatment period ($P<0.01$). Again, no complete tumor remissions were observed. Tumors exposed to rhuMAb HER-2 alone (rhuMAb) also showed reduced growth ($P<0.001$) as compared to controls (CON), but, again none achieved complete tumor remissions. In contrast, over the 9-week treatment period, the combined drug/antibody regimen produced a marked reduction in tumor volumes compared to control values ($P<0.001$), with one of six animals receiving this combination (rhuMAb/DOXO) achieving a complete tumor remission after 2–3 cycles of therapy, with partial remissions occurring in the remaining animals. Effects of combined drug-antibody therapy were significantly different from those found with antibody treatment alone ($P<0.01$). Although the magnitude of the combined doxorubicin-antibody effect is less than that found with cisplatin-antibody combinations (compare with Figure 6), this combination does provide a therapeutic advantage over treatment with either agent alone.

Effect of rhuMAb HER-2 in combination with chemotherapeutic drugs on unscheduled DNA synthesis

After demonstrating a clear therapeutic advantage of the combination of rhuMAb HER-2 and DNA-reactive drugs in HER-2-overexpressing cells, experiments were designed to evaluate possible mechanisms for this phenomenon. Previous work has shown that the cellular accumulation of cisplatin within cells is not affected by HER-2 antireceptor antibody in breast cancer cells (32). In addition, using methods previously described (Andrews *et al*., 1988), we find no significant effect of rhuMAb HER-2 at doses up to 100 μg/ml on

accumulation of [^{14}C]doxorubicin by MCF-7/HER-2 cells over 2 h (data not shown), indicating that the therapeutic advantage found with this combination also does not occur by altered cell accumulation of the anthracycline.

DNA repair is well known to play an important role in the recovery of cells from the toxicity of DNA-reactive drugs (Zhen *et al.*, 1992; Nielsen *et al.*, 1996), and changes in cisplatin-induced DNA repair have been reported to occur in HER-2-overexpressing cells after treatment with antibodies to HER-2 receptor (Pietras *et al.*, 1994; Arteaga *et al.*, 1994). To further evaluate the role of DNA repair as an explanation for the therapeutic advantage of antireceptor antibody and DNA-reactive drugs, we measured unscheduled DNA synthesis induced by cisplatin and doxorubicin in MCF-7 cells (Figure 8). As previously reported, treatment of breast cells with cisplatin alone elicits significant increases in unscheduled DNA synthesis as determined by thymidine incorporation into DNA (Pietras *et al.*, 1994). These data indicate an active DNA repair apparatus in MCF-7 parental, control and HER-2-overexpressing cells ($P<0.01$; Figure 8). Treatment with rhuMAb HER-2, however, significantly blocks this cisplatin-induced increase in DNA synthesis in MCF-7/HER-2 cells ($P<0.001$), but does not affect DNA repair in MCF-7 parental or control cells (Figure 8).

Although anthracyclines are not generally considered to be DNA-damaging agents, recent data suggests these agents may elicit indirect covalent modifications of DNA in mammary tissue (Purewal and Liehr, 1993; Nielsen *et al.*, 1996). To evaluate the potential effect of doxorubicin on DNA repair pathways, unscheduled DNA synthesis after doxorubicin in MCF-7 cells was also measured. Treatment of the breast cells with doxorubicin alone provoked a small, but measurable increase in unscheduled DNA synthesis ($P<0.01$; Figure 8). Treatment with rhuMAb HER-2 again significantly inhibits this doxorubicin-related increase in DNA repair in MCF/HER-2 cells. To confirm that this phenomenon was specifically due to HER-2 overexpression, it was tested in non-HER-2-overexpressing cells, i.e. parental and control MCF-7 cells. The drug-related effect on unscheduled DNA synthesis was not affected by antireceptor antibody in these cells, confirming the antibody specificity, interfering with DNA repair only in those cells overexpressing the HER-2 receptor.

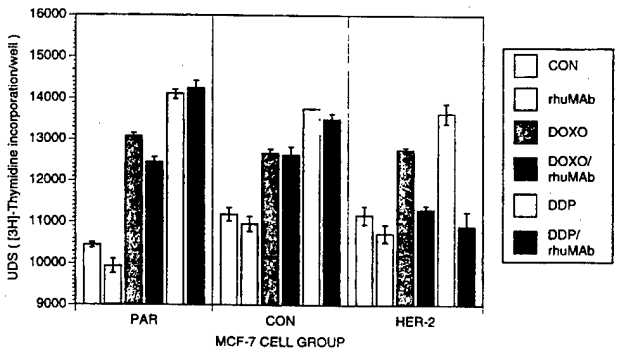

Figure 8 DNA repair (unscheduled DNA synthesis) in human MCF-7 breast carcinoma cells. Unscheduled DNA synthesis (UDS) was determined as described in Materials and methods. UDS was measured in MCF-7 parental (PAR), control (CON) and HER-2-overexpressing (HER-2) cells after treatment with control, rhuMAb HER-2 (200 μg/ml), cisplatin (DDP; 5 μM), doxorubicin (DOXO; 1 μM), 5 μM cisplatin in combination with 200 μg/ml rhuMAb HER-2 (DDP/rhuMAb), or 1 μM doxorubicin in combination with 200 μg/ml rhuMAb HER-2 (DOXO/rhuMAb). Doses selected for chemotherapeutic drugs were based on biologic dose-response data from preliminary experiments and from prior reports (Sawyer *et al.*, 1988; Pietras *et al.*, 1994; Boven *et al.*, 1996; Kurbacher *et al.*, 1996)

Effect of rhuMAb HER-2 in combination with cisplatin on formation and repair of cisplatin-induced DNA adducts in the DNA of MCF-7 cells with HER-2 overexpression

Since measures of unscheduled DNA synthesis provide only an indirect assessment of actual DNA repair, we sought to obtain direct data on the formation and removal of cisplatin-induced lesions in total genomic DNA of human breast cancer cells (Table 2). MCF-7/HER-2 cells were treated with 200 μM cisplatin for 1 h at 37°C, washed and then harvested at 0 or 20 h after the initial cisplatin treatment. To test the effect of antireceptor antibody, cells were first exposed to rhuMAb HER-2 (100 μg/ml) or control solution for 4 h prior to cisplatin treatment. After cisplatin exposure and cell washing, rhuMAb HER-2 was maintained in the culture medium at 100 μg/ml for the repair times indicated in Table 2. Table 2 shows data for the formation and removal of cisplatin-DNA adducts from the genomic DNA of cells treated with or without antireceptor antibody in three separate experiments. Treatment of HER-2-overexpressing MCF-7 cells with rhuMAb HER-2 prior to cisplatin promoted a significant reduction in the extent of DNA repair to 61% of that found in cells not treated with the antibody ($P<0.05$). This result confirms that rhuMAb HER-2 antibody is effective in blocking DNA repair of cisplatin-induced DNA adducts in human breast cancer cells with HER-2 overexpression.

Effect of time of administration of HER-2 antireceptor antibody on repair of cisplatin-damaged reporter DNA in human breast tumor cells

To test the hypothesis that the time of administration of HER-2 antireceptor antibody may be critical for

Table 2 Effect of recombinant humanized antibody to HER-2 on formation and repair of cisplatin adducts in the genome in human MCF-7 breast cancer cells with overexpression of HER-2 receptor

Therapy[a]	*Repair time (h)*	*Cisplatin-adducts (fmol/μg DNA)*	*% repair*
Without antibody	0	26.7±2.4	
	20	13.1±2.5	51
With antibody	0	29.4±1.7	
	20	20.2±1.5	31[b]

[a]Cells were treated with 200 μM cisplatin for 1 h in the presence of anti-HER-2 receptor antibody (rhuMAb HER-2) as outlined in the text. The time indicated is hours elapsed after cisplatin treatment. Per cent repair was estimated in three independent experiments as described elsewhere (Pietras *et al.*, 1994) using cisplatin adduct counts at 0 and 20 h as shown here. The latter counts were corrected for DNA replication using established methods (Jones *et al.*, 1991; Pietras *et al.*, 1994). [b]Significantly different from control at $P<0.05$ in three independent experiments

blockade of DNA repair, a CMV-driven β-galactosidase reporter plasmid was exposed to cisplatin *in vitro* and then transfected into MCF-7/HER-2 cells. At 24 h after transfection, the extent of repair was assayed by measuring reporter DNA expression in MCF-7/HER-2 cells that were incubated with rhuMAb HER-2 at 72 or 24 h prior to or at the end of the transfection (0 h). The transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase, to distinguish β-galactosidase-positive and -negative cells. In the presence of substrate, cells expressing bacterial β-galactosidase appeared blue and the percentage of stained cells was quantitated (see Figure 9). These data demonstrate that, as in the *in vivo* experiments above, antibody-modulated repair of cisplatin-damaged DNA is optimal when drug and antibody are administered in close temporal proximity. The timing of antibody cisplatin administration is critical and clearly affects the magnitude of observed responses in HER-2-overexpressing human breast cancer cells.

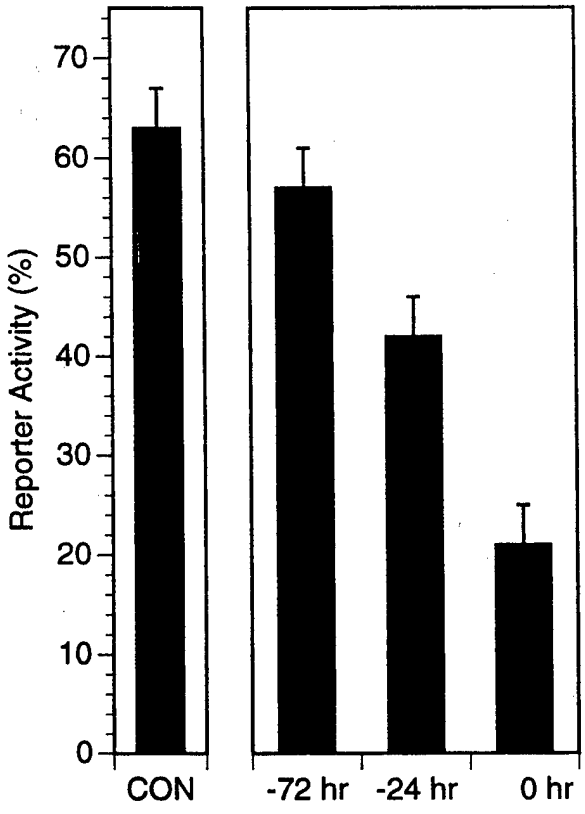

Figure 9 Time of administration of HER-2 antireceptor antibody affects repair of cisplatin-damaged reporter DNA in human breast cancer cells. CMV-driven β-galactosidase reporter plasmid was exposed to cisplatin *in vitro* and then transfected into MCF-7/HER-2 cells. At 24 h after transfection was completed, the extent of repair was assayed by measuring reporter DNA expression in MCF-7/HER-2 cells that were incubated without antibody (CON) or with rhuMAb HER-2 at 72 h or 24 h prior to transfection or at the end of the transfection (0 h). In each rhuMAb HER-2 group, cells were incubated with antibody for 2-h periods and were then washed and incubated further in the absence of antibody. Reporter activity is presented as the percentage of blue-stained cells in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase

Antireceptor antibodies disrupt regulation of p21/WAF1 expression in breast cancer cells with HER-2 overexpression.

The CDK inhibitor, p21/WAF1, is a critical mediator of the cellular response to DNA damage. We have assessed the activity of p21/WAF1 in response to cisplatin-induced DNA damage in MCF-7/HER-2 cells in the presence and in the absence of rhuMAb HER-2. Transcripts of p21/WAF1 were assessed by Northern blot analysis. MCF-7/HER-2 cells were treated with 100 μg/ml rhuMAb HER-2 alone or in combination with cisplatin treatment. Paired cells were treated with control solution or cisplatin alone. After 2 h or 24 h,

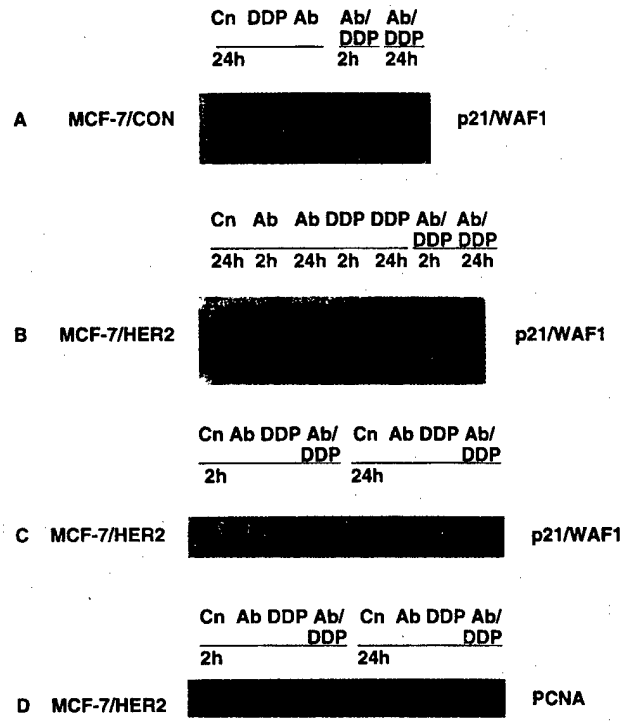

Figure 10 Monoclonal antibody to HER-2 growth factor receptor alters p21/WAF1 transcript and protein levels after cisplatin treatment of human breast cancer cells with HER-2 overexpression. After 2 h or 24 h, cells were processed for preparation of RNA or protein and determination of p21/WAF1 levels by established methods. (**a**) MCF-7/CON cells were treated with control solution (Cn), cisplatin (DDP, 5 μM), 100 μg/ml rhuMAb HER-2 (Ab) or rhuMAb HER-2 in combination with cisplatin (Ab/DDP) for 2 h or 24 h as indicated. Cell RNA was subjected to Northern blot analysis, with hybridization of the resulting blot with p21/WAF1 cDNA. The transcripts are predicted to be 2.1 kb, a size corresponding to that of human p21/WAF1 mRNA (El-Deiry *et al.*, 1993). (**b**) MCF-7/HER-2 cells were treated with control solution (Cn), 100 μg/ml rhuMAb HER-2 alone (Ab), cisplatin (DDP, 5 μM), or 100 μg/ml rhuMAb HER-2 in combination with cisplatin (Ab/DDP). Cell RNA was subjected to Northern blot analysis, with hybridization of the resulting blot with p21/WAF1 cDNA. (**c**) MCF-7/HER-2 cells were treated with control solution (Cn), 100 μg/ml rhuMAb HER-2 (Ab), cisplatin (DDP, 5 μM) or rhuMAb HER-2 in combination with cisplatin (Ab/DDP) for 2 h or 24 h as indicated. On Western blotting with anti-p21/WAF1 antibody, p21/WAF1 was found to occur as a 21-kd protein. (**d**) MCF-7/HER-2 cells were treated with control solution (Cn), 100 μg/ml rhuMAb HER-2 (Ab), cisplatin (DDP, 5 μM) or rhuMAb HER-2 in combination with cisplatin (Ab/DDP) for 2 h or 24 h as indicated. On Western blotting with anti-PCNA antibody, proliferating cell nuclear antigen was found to occur as a 36 kd protein. See text for additional details

cells were processed for preparation of RNA and determination of p21/WAF1 transcripts. The results show induction of p21/WAF1 transcripts at 2 h and 24 h after cisplatin treatment (Figure 10). However, increased levels of p21/WAF1 transcript are not sustained in MCF-7/HER-2 cells exposed to cisplatin in the presence of rhuMAb HER-2. Although the transcript level increases at 2 h, it is comparable to control levels of transcript by 24 h (Figure 10). The level of p21/WAF1 at 24 h is markedly less than the effect of cisplatin given without antibody. A reduction in the basal level of p21/WAF1 also occurred after 24 h exposure to antibody alone as compared with controls.

Western analyses of the level of p21/WAF1 protein in MCF-7/HER-2 cells likewise show enhanced amounts of the protein at 2 h and 24 h after cisplatin (Figure 10). However, as found in Northern studies, treatment of cells with antireceptor antibody elicits a reduced level of p21/WAF1 protein under basal conditions and blunts the anticipated response to chemotherapy at 24 h, as compared to controls. In contrast, the level of proliferating cell nuclear antigen (PCNA) is unchanged at 2 h and 24 h after cisplatin with or without rhuMAb HER-2 treatment (Figure 10). These results are consistent with independent reports on depletion of p21/WAF1 after withdrawal of growth factors (Canman *et al.*, 1995) and suggest an important role for growth factor pathways in modulating the activity of proteins which regulate DNA repair.

Discussion

HER-2 growth factor receptors which are overexpressed in approximately one-third of human breast and ovarian cancers are a logical target for the development of new therapeutic approaches which exploit the alteration. The current data demonstrate that a recombinant humanized anti-HER-2 receptor monoclonal antibody, similar to the murine antibody 4D5 from which it was derived (Hudziak *et al.*, 1989; Fendly *et al.*, 1990), inhibits growth of HER-2-overexpressing human breast and ovarian tumor xenografts in athymic mice. In addition, the magnitude of growth inhibition is directly related to dose of rhuMAb HER-2, with the highest dose tested showing a 10–14-fold decrease in tumor size compared to control. These data provide strong evidence for an *in vivo* antiproliferative effect of rhuMAb HER-2 in tumors derived from cells which overexpress HER-2 protein. In addition, they are consistent with the initial observations which demonstrated that monoclonal antibodies against the extracellular domain of the HER-2 receptor can suppress tumorigenesis of HER-2-transformed NIH3T3 and NR6 cells (Drebin *et al.*, 1988; Chazin *et al.*, 1992) as well as inhibit the growth of human breast carcinoma cells overexpressing the HER-2 gene product *in vitro* (Hudziak *et al.*, 1989). The growth inhibitory effects of antibody alone, however, are cytostatic, with tumor growth recurring after discontinuation of antibody administration.

In view of earlier reports by Aboud-Pirak *et al.* (1988) and the subsequent studies of our and other laboratories (Christen *et al.*, 1991; Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1994; Arteaga *et al.*, 1994; Dixit *et al.*, 1997) indicating potentiation of tumor cell cytotoxic effects using antireceptor antibody and chemotherapeutic agents, therapy with antibody in combination with cisplatin or doxorubicin was tested in the current study. The present *in vivo* data confirm the considerable potentiation of cisplatin cytotoxicity and some potentiation of anthracycline cytotoxicity by combined treatment with rhuMAb HER-2 in human breast cancer cells which overexpress the HER-2 receptor. The effect is especially pronounced when multiple cycles of combined treatment are administered, with up to a 1000-fold therapeutic difference in cisplatin/antibody therapy and a 200-fold difference in doxorubicin/antibody therapy. The therapeutic advantage of combined treatment with antibody and cisplatin is clearly evident since tumor remissions were found which could not be achieved when either agent was administered alone at sublethal doses (Berenbaum, 1989; Wampler *et al.*, 1992). Using a formal median-effects approach (Chou and Talalay, 1984), a true synergistic decrease in human cancer cell growth *in vitro* and *in vivo* by combination therapy with cisplatin and the anti-HER-2 antibody has been shown (Pietras *et al.*, 1994). The current study also demonstrates that timing of antireceptor antibody and cisplatin administration is critical in promoting an optimal *in vivo* antitumor effect. Treatment with cisplatin and rhuMAb HER-2 in relatively close temporal proximity appears necessary for greatest suppression of human breast tumor growth, with optimum inhibition occurring when the antibody is given shortly before or simultaneously with cisplatin. The profound antitumor toxicity of cisplatin together with rhuMAb HER-2 administered in repeated therapy as detailed here supports the use of these agents in combination over multiple courses.

Although the molecular consequences of cisplatin (Chu, 1994) and doxorubicin (Sawyer *et al.*, 1988; Purewal and Liehr, 1993; Cutts *et al.*, 1994; Nielsen *et al.*, 1996) therapy and antireceptor antibody-receptor interactions (Drebin *et al.*, 1988; Sarup *et al.*, 1991; Scott *et al.*, 1991) are incompletely understood, the present evidence is consistent with independent reports which show that antibodies to the HER-2 receptor not only elicit growth inhibition on their own (Drebin *et al.*, 1988; Hudziak *et al.*, 1989; Chazin *et al.*, 1992) but can modulate the sensitivity to DNA-reactive drugs (Hancock *et al.*, 1991; Shepard *et al.*, 1991; Pietras *et al.*, 1994). Doxorubicin is generally considered to act as a DNA-intercalating agent, but recent reports suggest that anthracyclines might also indirectly promote covalent modification of DNA and possibly induce adduct formation (Sawyer *et al.*, 1988; Purewal and Liehr, 1993; Cutts *et al.*, 1994). Cisplatin tends to produce intrastrand adducts and interstrand crosslinks in DNA and also evokes changes in the expression and association of certain sequence-specific binding proteins with damaged DNA (Chu, 1994). Unlike doxorubicin, however, a significant role of DNA repair has been well-established in the recovery of cells from the toxicity of cisplatin (Chu, 1994). Cells which incur DNA damage exhibit cell cycle delays, and these delays are considered to be critical to allow repair of DNA before continuing through the cell cycle to

mitosis (Sorenson *et al.*, 1990). Miscommunication in these complex signal pathways, perhaps due to antireceptor antibody or to inappropriate ligand stimulation (Kinzel *et al.*, 1990), could lead to lethal consequences for the cell. Similarly, tyrosine kinase inhibitors which preferentially suppress HER-2 kinase have been found to sensitize HER-2-overexpressing lung cancer cells to anticancer drugs that damage DNA (Zhang and Hung, 1996; Tsai *et al.*, 1996). Another link between receptor signal transduction pathways and cisplatin sensitivity has been found to occur on modulation of protein kinase C activity (Hofman *et al.*, 1988; Isonishi *et al.*, 1990), an enzyme involved in signal transduction to the nucleus (Olson *et al.*, 1993). This signal pathway is known to be down-regulated by long exposure of breast cancer cells to the 4D5 anti-HER-2 antibody (Hancock *et al.*, 1991; Sarup *et al.*, 1991). It is clear that further mechanistic study of this phenomenon is required to render a full biologic explanation for growth factor receptor-chemotherapeutic drug interactions and the *in vivo* schedule-dependency of this effect.

In p21/WAF1 −/− cancer cells, p21 WAF1 deficiency is associated with a prominent defect in DNA repair (McDonald *et al.*, 1996). Although induction of the cyclin-dependent kinase inhibitor, p21/WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism involving the tumor suppressor protein p53 (El-Diery *et al.*, 1993), recent work suggests that growth factors may provide an alternative pathway for regulation of p21/WAF1 expression. In the case of growth factor stimulatory pathways, induction of p21/WAF1 appears not to require p53 and may be activated instead by mitogen-activated protein kinase (Liu *et al.*, 1996). Withdrawal of growth factors *in vitro* has also been associated with down-regulation of p21/WAF1 expression and with enhanced cell killing in response to DNA damage. The present work provides further evidence that the growth factor receptor, HER-2, can modulate DNA damage response pathways in breast cancer cells (Pietras *et al.*, 1994; Arteaga *et al.*, 1994) and suggests that this cross-communication may involve modulation of p21/WAF1. Others have reported recently that heregulin, a natural ligand to HER-2/HER-3 heterodimers, promotes the tyrosine phosphorylation of HER-2 receptor and the increased expression of p21/WAF1 in MCF-7 cells with HER-2 overexpression (Bacus *et al.*, 1996). We have assessed the activity of p21/WAF1 in response to DNA damage in MCF-7/HER-2 cells in the presence and in the absence of rhuMAb HER-2 antibody. Transcripts of p21/WAF1 showed significant induction at 2 h and 24 h after cisplatin treatment. However, increased levels of p21/WAF1 transcript were not sustained in MCF-7/HER-2 cells exposed to chemotherapy in the presence of rhuMAb HER-2. The level of p21/WAF1 at 24 h is less than the effect of cisplatin given without antibody. A notable reduction in the basal level of p21/WAF1 also occurred after 24 h exposure to antibody alone as compared with controls. These results and independent reports on depletion of p21/WAF1 after withdrawal of growth factors (Canman *et al.*, 1995) suggest an important role for growth factor pathways in modulating the activity of proteins which regulate DNA repair (Tsai *et al.*, 1996). Dysregulation of p21/WAF1 occurs after treatment with antireceptor antibody, and this event appears to adversely influence the cell response to DNA damage.

Future work will be required to test the hypothesis that mitogen-activated protein kinase or other components of the HER-2 signaling pathway (Tsai *et al.*, 1996; Lewis *et al.*, 1996; Yen *et al.*, 1997) play a role in the regulation of p21/WAF1. On binding of heregulin ligand, transient phosphorylation of HER-2 protein occurs, and this promotes downstream activation of MAP kinase (Marte *et al.*, 1995; Reese and Slamon, 1997). In contrast, antibodies to HER-2 receptor generally induce prolonged phosphorylation and down-regulation of HER-2 protein and disrupt the association of HER-2 with HER-3 (Sarup *et al.*, 1991; Graus-Porta *et al.*, 1995; Marte *et al.*, 1995; Reese and Slamon, 1997). Some anti-HER-2 receptor antibodies act as partial agonists and promote weak or no activation of MAP kinase. As anti-HER-2 antibodies, tyrosine kinase inhibitors with specificity for HER-2 kinase are also known to enhance the sensitivity of HER-2-overexpressing cancer cells to DNA-damaging agents (Aboud-Pirak *et al.*, 1989; Tsai *et al.*, 1996). Although the activity of rhuMAb HER-2 remains to be fully characterized, downstream effects of HER-2 stimulation, such as activation of MAP kinase, are likely to be affected by rhuMAb HER-2.

Significant data support our hypothesis that p21/WAF1 may play a vital role in mediating rhuMAb HER-2 effects on DNA damage pathways in the breast cancer cell. However, alterations in other regulatory proteins, including p53, MDM2 and GADD45, may also contribute to the process observed in the present work (Kastan *et al.*, 1991; Chen *et al.*, 1994; Canman *et al.*, 1995). Nevertheless, the available evidence suggests that pathways of DNA replication, DNA repair and DNA degradation may have common regulatory elements, with the final outcome at a cellular level dependent on the extent of DNA damage (Wu and El-Deiry, 1996). Growth factor receptors are likely to play a significant regulatory role in this process, and manipulation of this pathway in the clinic with rhuMAb HER-2 may provide therapeutic benefit to patients with HER-2-overexpressing breast cancer.

A further aspect of the present findings is the possibility that HER-2 overexpression is linked to genesis of resistance to chemotherapeutic agents. Development of the drug-resistant, metastatic phenotype is responsible for the bulk of treatment failures in breast cancer (Harris *et al.*, 1992), and involvement of oncogenes in drug resistance was proposed by Scanlon *et al.* (1989). Further evidence in support of this hypothesis has been published (Isonishi *et al.*, 1991; Benz *et al.*, 1993). The potential role of HER-2 proto-oncogenes in modulation of chemotherapeutic drug sensitivity has been suggested from retrospective analysis of results of several therapeutic clinical studies (Allred *et al.*, 1992; Gusterson *et al.*, 1992; Muss *et al.*, 1994) and from limited laboratory studies (Benz *et al.*, 1993; Tsai *et al.*, 1993). If correct, these findings could have important implications in patient management and treatment decisions. Assessment of HER-2 receptor overexpression already provides additional prognostic information in patients with both node-positive (Slamon *et al.*, 1987; 1989a; Van Diest *et al.*, 1992) and node-negative (Ro *et al.*, 1989; Press *et al.*,

1993; Seshadri *et al.*, 1993) breast cancer. Clues for the influence of HER-2 signaling pathways on chemotherapeutic drug resistance require extension of clinical and laboratory investigations similar to those already reported (Benz *et al.*, 1993; Tsai *et al.*, 1993; Pegram *et al.*, 1997).

Treatment of human cancers requires new approaches designed to minimize toxicity to normal cells and maximize damage to tumor targets. Therapy directed at specific alterations unique to the tumor cell should prove more rational, less toxic and potentially more therapeutic. Remission of human HER-2-overexpressing breast tumors in nude mice after combined therapy with cisplatin and rhuMAb HER-2 offers the potential to achieve such a goal. This phenomenon, which we have termed receptor-enhanced chemosensitivity (REC; Pietras *et al.*, 1994) has already been implemented in ongoing phase II–III clinical combination chemotherapy trials in human subjects (Pegram *et al.*, 1995). The potential specificity of the therapeutic use of anti-HER-2 antibodies to alter DNA repair in such a way as to specifically render HER-2 overexpressing cells more sensitive to certain drugs is bolstered by the present findings and by independent reports showing little to no reactivity of such antibodies with most normal or non-overexpressing cells (Press *et al.*, 1990; Pietras *et al.*, 1994). This should allow us to exploit the overexpression of the HER-2 gene in many breast and ovarian cancers to develop new and more rational approaches to the therapy of these diseases. In view of some of the potential obstacles and costs to long-term monoclonal antibody therapies in human cancer, an alternative therapeutic use of antireceptor antibodies may be in combination with cytotoxic agents to achieve optimal cytocidal effects rather than cytostasis.

Materials and methods

Cell lines and cell culture

The well-characterized human breast carcinoma cell line, MCF-7, and the human ovarian carcinoma cell line designated CAOV3 were obtained from American Type Culture Collection (Rockville, MD). All cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM freshly added glutamine and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA).

Transfections and amplification/overexpression of human HER-2 gene in human cells

Human ovarian CAOV3 and breast MCF-7 carcinoma cells with normal levels of HER-2 gene expression were transfected with full-length cDNA of the human HER-2 gene. The latter was cloned from a primary human breast cancer specimen and characterized previously in our laboratory (Slamon *et al.*, 1987, 1989b; Chazin *et al.*, 1992). The vector for introduction of HER-2 gene into human cells contained the full-length human HER-2 gene coding sequence ligated into the replication-defective retroviral expression vector, pLXSN (Miller and Rosman, 1989; Chazin *et al.*, 1992). This was achieved by ligating a 3.8 kb *Nco*I to *Mst*II fragment containing the full HER-2 coding sequence, without the polyadenylation signal, into an amphotrophic retroviral expression vector with a Moloney murine leukemia virus (MMLV) promoter, a neomycin phosphotransferase gene and a packaging signal, but devoid of viral protein coding sequences; thus rendering the virus replication-defective. The pLXSN construct has an extended packaging signal for high virus titre as well as a mutated *gag* start codon and a shortened envelope region to decrease the risk of helper virus generation (Miller and Rosman, 1989; Chazin *et al.*, 1992). Virus-producing cells were prepared by a transient rescue procedure as described before (Miller and Rosman, 1989; Chazin *et al.*, 1992). As noted above, this vector also contains a neomycin resistance gene (neomycin phosphotransferase) which confers cellular resistance to the aminoglycoside antibiotic G418, thus allowing selection of primary infectants. The pLXSN vector devoid of HER-2 sequences (designated CON) but containing the neomycin phosphotransferase gene was packaged in an identical fashion and served as a retroviral control in appropriate experiments. Ovarian and breast carcinoma cells were infected as previously described (Chazin *et al.*, 1992). Cell lines established by this method of gene transfer were characterized at the DNA, RNA, protein and immunohistochemical level for copy number and expression level of HER-2 gene as reported elsewhere (Slamon *et al.*, 1987; Chazin *et al.*, 1992).

Tumor formation in nude mice

Breast and ovarian cells were injected subcutaneously at $4-5\times10^7$ cells/animal in the mid-back region of female athymic mice (20–25 gm). Mice from an inbred Swiss nude strain and from an outbred CD1 nu/nu strain (Charles River, Cambridge, MA) were used. Mice were maintained and handled under aseptic conditions. Animals were allowed free access to food and water throughout the study. Prior to tumor cell innoculation, all mice were primed for 7 days with 17β-estradiol introduced subcutaneously in a biodegradable carrier-binder (1.7 mg estradiol/pellet; Innovative Research of America, Inc.). A period of 7 to 14 days elapsed to allow formation of tumor nodules. Animals were then randomized into uniform groups based on animal weight and tumor volume at the start of the experiment. Animals (5–7 mice/group) were treated via i.p. injection. Animals received either an isotype-matched IgG1 control antibody, the murine 4D5 antibody, the rhuMAb 4D5 HER-2 antibody, cisplatin (DDP, cis-diammine-dichloroplatinum(II); Bristol-Meyers, Squibb), doxorubicin or a combination treatment of the above as designated in the results section. Tumor nodules were monitored by micrometer measurements, with tumor volume calculated as the product of length × width × height. Tumor tissue was analysed for HER-2 receptor expression by established immunohistochemical methods (Slamon *et al.*, 1987; Chazin *et al.*, 1992).

Monoclonal antibodies

Anti-HER-2 receptor monoclonal antibody 4D5 (2.5 mg/ml; Lot No.G088AL/S9839AX) was prepared as previously described (Fendly *et al.*, 1990). Methods for construction of a humanized form of 4D5 containing only the antigen-binding loops from murine 4D5 and human variable region framework residues plus IgG1 constant domains (rhuMAb HER2 at 5.15 mg/ml; Lot No.GN1450/M3-RD168) were reported elsewhere (Carter *et al.*, 1992). Human IgG1 (5.3 mg/ml) was used as control solution in appropriate experiments. Our choices for dose and schedule of therapy were based on results of prior pharmacokinetic studies using murine monoclonal antibody 4D5 and rhuMAb HER-2 (Maneval *et al.*, 1991; DeSantes *et al.*, 1992; Pietras *et al.*, 1994). These data showed that measures of serum clearance and permanence times in serum are similar for the humanized and native murine monoclonal

antibodies. Maintenance of a serum antibody concentration in the range of 10 μg/ml required a dose of >2 mg/kg mouse body weight given every 4 days. Although time variant processes such as production of an antiglobulin response (mouse anti-human antibody) can occur in these systems, this effect has not been observed in studies with the rhuMAb HER-2 antibody. In athymic mice receiving twice-weekly i.p. doses of humanized antibody for 7 weeks, no enhanced immune clearance of humanized HER-2 antibody and no anti-humanized MAb antibodies have been measured in athymic mouse serum samples. Athymic mice were randomized to receive low (1–3 mg/kg/dose) or high (10–100 mg/kg/dose) doses of rhuMAb HER-2. Equal volumes of the agents were given.

Unscheduled DNA synthesis

Unscheduled DNA synthesis, DNA repair which is nonsemiconservative in nature, was determined by established methods (Pietras *et al*., 1994). Cell monolayers were preincubated with or without antibody in arginine-deficient, reduced serum (0.5%) media for 5 h, followed by exposure to hyroxyurea for 1 h. Cells were then treated with cisplatin or doxorubicin (in the presence of hydroxyurea) for 1 h and finally incubated with [^{3}H]thymidine and hydroxyurea for 3 h. Cell groups were harvested, and cellular DNA was bound to glass fiber filters and collected for liquid scintillation counting of [^{3}H]thymidine incorporation/group.

Detection of genomic cisplatin adducts

Cells were cultivated *in vitro* to 60–70% confluence. For 12 h prior to the start of the experiment, cells were labeled with ^{3}H-thymidine at 0.1 μCi/ml in order to provide a correction factor for any cellular replication during the course of the experiment (Jones *et al*., 1991; Pietras *et al*., 1994). Thereafter, cells were incubated in fresh medium with rhuMAb HER-2 at 200 μg/ml or control solution for 4 h. The cells were then exposed to 200 μM cisplatin (freshly made) for 1 h, washed in cisplatin-free media and harvested at 0 and 20 h after the cisplatin treatment. Cells treated with or without rhuMAb HER-2 were maintained in the same media after removal of the drug. Harvested cells were pelleted and stored at −20°C until DNA isolation. DNA was isolated and prepared as described before (Pietras *et al*., 1994). Total platinum content was assessed by atomic absorption spectrometry using a Perkin-Elmer Zeeman spectrometer (Zhen *et al*., 1992).

In vivo *repair of reporter DNA damaged by cisplatin*

Introduction of cisplatin-damaged reporter DNA into breast tumor cells was carried out by established methods. Prior to transfection, CMV-driven β-galactosidase (pCMV-β; Clontech), a reporter DNA, was prepared without or with exposure to cisplatin *in vitro* as before (McDonald *et al*., 1996). For transfection experiments, cells were plated 72 h prior to transfection, and transfections with internal controls for transfection efficiency were carried out as described previously (McDonald *et al*., 1996). In these transfection experiments, 1.5 μg undamaged or cisplatin-damaged DNA was used. At 24 h after transfection, the extent of repair was assayed by measuring reporter DNA expression. The transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase, to distinguish β-galactosidase-positive and -negative cells. In the presence of substrate, cells expressing bacterial β-galactosidase appeared blue and the percentage of stained cells was quantitated.

Measurement of p21/WAF1 transcript and protein levels

Transcripts of p21/WAF1 were determined by Northern blot analysis, using a protocol as before (Slamon *et al*., 1987; 1989a; El-Diery *et al*., 1993; Pietras *et al*., 1995). In brief, breast cancer cells with and without HER-2 overexpression were treated with or without rhuMAb HER-2 for 4 h before exposure to chemotherapy. Cells were then maintained for 2 h and 24 h prior to harvesting and processing for collection of RNA. After Northern blot analysis, the resulting blots were hybridized with p21/WAF1 cDNA (generously provided by Dr Bert Vogelstein).

Western analyses of the level of p21/WAF1 protein in breast cancer cells were conducted with methods as before (Pietras *et al*., 1995). We assessed p21/WAF1 protein in response to DNA damage in breast cancer cells in the presence and in the absence of growth factor receptor antibody. Breast cancer cells with and without HER-2 overexpression were treated with 100 μg/ml rhuMAb HER-2 for 4 h before exposure to cisplatin. Cells were then maintained for 2 h and 24 h prior to harvesting and processing of cell lysates for electrophoresis (Pietras *et al*., 1995). Immunoblotting was done with monoclonal antibody 6B6 with specificity for human p21/WAF1 (Pharmingen). In other studies, immunoblotting was also done with monoclonal antibody to proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology).

Statistical analyses

Analysis of variance (ANOVA) was conducted on tumor size data at each time point. In each group, only data from animals surviving through day 21 were included in statistical assessments. Average tumor size in each treated group was compared to that in the appropriate control group via a two-tailed *t*-test using the pooled error variance from the ANOVA (Campbell, 1976).

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References

Aaronson SA. (1991). *Science*, **254**, 1146–1153.

Aboud-Pirak E, Hurwitz E, Pirak ME, Bellot F, Schlessinger J and Sela M. (1988). *J. Natl. Cancer Inst.*, **80**, 1605–1611.

Aboud-Pirak E, Hurwitz E, Bellot F, Schlessinger J, and Sela M. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 3778–3781.

Allred DC, Clark GM, Tandon AK, Molina R, Tormey DC, Osborne CK, Gilchrist KW, Mansour EG, Abeloff M, Eudey L and McGuire WL. (1992). *J. Clin. Oncol.*, **10**, 599–605.

Andrews PA, Velury S, Mann SC and Howell SB. (1988). *Cancer Res.*, **48**, 68–73.

Arteaga CL, Winnier AR, Poirier MC, Lopez-Larraza DM, Shawver LK, Hurd SD and Stewart SJ. (1994). *Cancer Res.*, **54**, 3758–3765.

Bacus SS, Yarden Y, Oren M, Chin DM, Lyass L, Zelnick CR, Kazarov A, Toyofuku W, Gray-Bablin J, Beerli RR, Hynes NE, Nikiforov M, Haffner R, Gudkov A and Keyomarsi K. (1996). *Oncogene*, **12**, 2535–2547.

Bargmann CI, Hung MC and Weinberg RA. (1986). *Cell*, **45**, 649–657.

Benz C, Scott G, Sarup J, Johnson R, Tripathy D, Coronado E, Shepard H and Osborne C. (1993). *Breast Cancer Res. Treatment*, **24**, 85–95.

Berenbaum MC. (1989). *Pharmacol. Rev.*, **19**, 93–138.

Boven E, deJong J, Kuiper C, Bast A and van der Vijgh W. (1996). *Eur. J. Cancer*, **32A**, 1382–1387.

Campbell RC. (1976). *Statistics for Biologists*. University Printing House, Cambridge.

Canman CE, Gilmer TM, Coutts S and Kastan M. (1995). *Genes & Development*, **9**, 600–611.

Carter P, Presta L, Gorman CM, Ridgway JBB, Henner D, Wong WLT, Rowland AM, Knotts C, Carver ME and Shepard HM. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 4285–4289.

Chazin VR, Kaleko M, Miller AD and Slamon DJ. (1992). *Oncogene*, **7**, 1859–1866.

Chen C, Oliner J, Zhan Q, Fornace A, Vogelstein B and Kastan M. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 2684–2688.

Chou T-C and Talalay P. (1984). *Adv. Enz. Reg.*, **22**, 27–43.

Christen RD, Hom DK, Porter DC, Andrews PA, MacLeod CL, Hafstrom L and Howell SB. (1991). *J. Clin. Invest.*, **86**, 1632–1640.

Chu G. (1994). *J. Biol. Chem.*, **269**, 787–790.

Coussens L, Yang-Feng TC, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Lieberman TA, Schlessinger J, Francke U, Levinson A and Ullrich A. (1985). *Science*, **230**, 1132–1139.

Cutts SM, Parsons PG, Sturm RA and Phillips DR. (1994). *J. Biol. Chem.*, **271**, 5422–5429.

DeSantes K, Slamon DJ, Anderson S, Shepard M, Fendly B, Maneval D and Press O. (1992). *Cancer Res.*, **52**, 1916–1923.

DiFiore P, Pierce J, Kraus MH, Segatto O, King CR and Aaronson SA. (1987). *Science*, **237**, 178–181.

Dixit M, Yang JL, Poirier MC, Price JO, Andrews PA and Arteaga CL. (1997). *J. Natl. Cancer Inst.*, **89**, 365–373.

Drebin JA, Link VC and Greene MI. (1988). *Oncogene*, **2**, 387–394.

El-Diery W, Tokino T, Velculescu V, Levy D, Parsons R, Trent J, Lin D, Mercer W, Kinzler K and Vogelstein B. (1993). *Cell*, **75**, 817–825.

Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA and Ullrich A. (1990). *Cancer Res.*, **50**, 1550–1558.

Graus-Porta D, Beerli R and Hynes N. (1995). *Mol. Cell. Biol.*, **15**, 1182–1191.

Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, Styles J, Rudenstam C-M, Golouh R, Reed R, Martinez-Tello F, Tiltman A, Torhorst J, Grigolato P, Bettelheim R, Neville AM, Burki K, Castiglione M, Collins J, Lindtner J and Senn H-J. (1992). *J. Clin. Oncol.*, **10**, 1049–1056.

Hancock MC, Langton BC, Chan T, Toy P, Monahan JJ, Mischak RP and Shawver LK. (1991). *Cancer Res.*, **51**, 4575–4580.

Harris J, Lippman M, Veronesi U and Willett W. (1992). *N. Engl. J. Med.*, **327**, 473–480.

Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM and Ullrich A. (1989). *Mol. Cell. Biol.*, **9**, 1165–1172.

Hudziak RM, Schlessinger J and Ullrich A. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 7159–7163.

Hofman J, Doppler W, Jakob A, Maly K, Posch L, Uberall F and Grunicke H. (1988). *Int. J. Cancer*, **42**, 382–388.

Isonishi S, Andrews P and Howell S. (1990). *J. Biol. Chem.*, **265**, 3623–3627.

Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A and Howell SB. (1991). *Cancer Res.*, **51**, 5903–5909.

Jensen L and Linn S. (1988). *Mol. Cell. Biol.*, **8**, 3964–3969.

Jones JC, Zhen W, Reed E, Parker RJ, Sancar A and Bohr VA. (1991). *J. Biol. Chem.*, **266**, 7101–7107.

Kastan M, Onyekwere O, Sidransky D, Vogelstein B and Craig R. (1991). *Cancer Res.*, **51**, 6304–6311.

Kinzel V, Kaszkin M, Blume A and Richards J. (1990). *Cancer Res.*, **50**, 7932–7936.

Kraus MH, Popescu NC, Amsbaugh SC and King CR. (1987). *EMBO J.*, **6**, 605–610.

Kurbacher CM, Wagner U, Kolster B, Andreotti PE, Krebs D and Bruckner HW. (1996). *Cancer Letters*, **103**, 183–189.

Lemoine NR, Staddon S, Dickson C, Barnes DM and Gullick WJ. (1990). *Oncogene*, **5**, 237–239.

Lewis G, Lofgren J, McMurtrey A, Nuijens A, Fendly B, Bauer K and Sliwkowski M. (1996). *Cancer Res.*, **56**, 1457–1465.

Liu Y, Martindale J, Gorospe M and Holbrook N. (1996). *Cancer Res.*, **56**, 31–35.

Lofts FJ and Gullick WJ. (1992). *Cancer Treatment and Res.*, **61**, 161–179.

Maneval D, Mordenti J, Hutchins B, Scates S, Hansen S, Keith D, Kotts C, Fletcher B, Fendly B, Blank G, Venerlein D, Slamon D, Shepard M and Green J. (1991). *J. Nucl. Med.*, **32**, 1837–1842.

Marte B, Graus-Porta D, Jeschke M, Fabbro D, Hynes N and Taverna D. (1995). *Oncogene*, **10**, 167–175.

McDonald III ER, Wu GS, Waldman T and El-Deiry WS. (1996). *Cancer Res.*, **56**, 2250–2255.

Mendelsohn J and Fan Z. (1997). *J. Natl. Cancer Inst.*, **89**, 341–343.

Miller AD and Rosman GJ. (1989). *Biotechniques*, **7**, 980–990.

Muss HB, Thor AD, Berry DA, Kute T, Liu ET, Koerner F, Cirrincione CT, Budman DR, Wood WC, Barcos M and Henderson IC. (1994). *N. Engl. J. Med.*, **330**, 1260–1266.

Nielsen D, Maare C and Skovsgaard T. (1996). *Gen. Pharmacol.*, **27**, 251–255.

Olson EN, Burgess R and Staudinger J. (1993). *Cell Growth Differ.*, **4**, 699–705.

Pauletti G, Godolphin W, Press MF and Slamon DJ. (1996). *Oncogene*, **13**, 63–72.

Pegram M, Lipton A, Pietras R, Hayes D, Weber B, Baselga J, Tripathy D, Twadell T, Glaspy J and Slamon DJ. (1995). *Proc. Am. Soc. Clin. Oncol.*, **14**, 106.

Pegram M, Finn RS, Arzoo K, Beryt M, Pietras R and Slamon D. (1997). *Oncogene*, **15**, 537–547.

Pierce JH, Arnstein P, DiMarco E, Artrip J, Kraus MH, Lonardo F, DiFiore P and Aaronson SA. (1991). *Oncogene*, **6**, 1189–1194.

Pietras RJ, Fendly B, Chazin V, Pegram M, Howell S and Slamon D. (1994). *Oncogene*, **9**, 1829–1838.

Press MF, Cordon-Cardo C and Slamon DJ. (1990). *Oncogene*, **5**, 953–962.

Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowicz M, Danyluk J, Godolphin W Sliwkowski M, Akita R, Paterson MC and Slamon DJ. (1993). *Cancer Res.*, **53**, 4960–4970.

Purewal M and Liehr JG. (1993). *Cancer Chemother. Pharmacol.*, **33**, 239–244.

Reese D and Slamon DJ. (1997). *Stem Cells*, **15**, 1–8.

Ro J, El-Naggar A, Ro JY, Blick M, Frye D, Fraschini G, Fritsche H and Hortobagyi G. (1989). *Cancer Res.*, **49**, 6941–6944.

Sarup JC, Johnson RM, King KL, Fendly BM, Lipari MT, Napier MA, Ullrich A and Shepard HM. (1991). *Growth Regulation*, **1**, 72–82.

Sawyer TW, Gill RD, Smith-Oliver T, Butterworth BE and DiGiovanni JD. (1988). *Carcinogenesis*, **9**, 1197–1202.

Scanlon K, Kashani-Sabet M, Miyachi H, Sowers L and Rossi J. (1989). *Anticancer Res.*, **9**, 1301–1312.

Scott GK, Dodson J, Montgomery P, Johnson R, Sarup J, Wong W, Ullrich A, Shepard H and Benz C. (1991). *J. Biol. Chem.*, **266**, 14300–14305.

Semba K, Kamata N, Toyoshima K and Yamamoto T. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 6479–6502.

Seshadri R, Firgaira FA, Horsfall DJ, McCaul K, Setlur V and Kitchen P. (1993). *J. Clin. Oncol.*, **11**, 1936–1942.

Shepard HM, Lewis G, Sarup J, Fendly B, Maneval D, Mordenti J, Figari I, Kotts C, Palladino M, Ullrich A and Slamon DJ. (1991). *J. Clin. Immunol.*, **11**, 117–127.

Slamon DJ, Clark GM, Wong S, Levin W, Ullrich A and McGuire W. (1987). *Science*, **235**, 177–182.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A and Press MF. (1989a). *Science*, **244**, 707–712.

Slamon DJ, Press MF, Godolphin W, Ramos L, Haran P, Shek L, Stuart SG and Ullrich A. (1989b). *Cancer Cells*, **7**, 371–378.

Sorenson CM, Barry MA and Eastman A. (1990). *J. Natl. Cancer Inst.*, **82**, 749–755.

Soule HD and McGrath CM. (1980). *Cancer Letters*, **10**, 177–189.

Tsai C-M, Chang K-T, Perng R-P, Mitsudomi T, Chen M-H, Kadoyama C and Gazdar AF. (1993). *J. Natl. Cancer Inst.*, **85**, 897–901.

Tsai C-M, Levitzki A, Wu L-H, Chang K-T, Cheng C-C, Gazit A and Perng R-P. (1996). *Cancer Res.*, **56**, 1068–1074.

Van Diest P, Baak J, Matze-Cok P and Bacus S. (1992). *Pathol. Res. Prac.*, **188**, 344–350.

Wampler G, Carter W, Campbell E and Keefe P. (1992). *Cancer Chemother. Pharmacol.*, **31**, 111–117.

Wu GS and El-Deiry S. (1996). *Clin. Cancer Res.*, **2**, 623–633.

Yarden Y and Ullrich A. (1988). *Annu. Rev. Biochem.*, **57**, 443–478.

Yen L, Zeng-Rong N, You X-L, Richard S, Langton-Webster BC and Alaoui-Jamali MA. (1997). *Oncogene*, **14**, 1827–1835.

Zhang L and Hung M-C. (1996). *Oncogene*, **12**, 571–576.

Zhen W, Link CJ, O'Connor PM, Reed E, Parker R, Howell SB and Bohr VA. (1992). *Mol. Cell. Biol.*, **12**, 3689–3698.

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HER-2 TYROSINE KINASE PATHWAY REGULATES ESTROGEN RECEPTOR AND GROWTH IN HUMAN BREAST CANCER CELLS

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Breast cancer is a disease that will affect 1 of 8 women in the United States. Currently, 2.6 million women are living with breast cancer. This disease is caused by the uncontrolled division of breast cells which can spread into and destroy normal tissues. Growth of breast cells is normally regulated by hormones such as estrogen and by peptide growth factors which bind to specific receptors at the external surface of the cell. These receptors telegraph growth-promoting signals to specific genes in the nucleus of the cell. Changes in cancer-related genes can lead to the production of many extra copies of growth factor receptors. These excess receptors then signal for non-stop cell division. A new biologic approach to cancer therapy involves efforts to cut the communication lines between these receptors and the cell nucleus, thus slowing or blocking cell division. Antiestrogen therapy is one example of this approach, and it is often used to treat breast cancer. Unfortunately, most patients eventually become resistant to antiestrogens. This failure of antihormone therapy may be due, in part, to the presense of excess receptors for growth factors. Our research work has revealed mechanisms by which surplus receptors for growth factors may affect the hormone sensitivity of breast cancers. This new information has led to the development of novel treatments that may prove more effective in blocking growth-promoting signals. Further understanding of the complex interactions between estrogen and growth factor receptors may help to guide patient management decisions and lead us to improved treatments to prevent the progression of human breast cancer.

Keywords: Estrogen Receptor, HER-2/neu, Erb B2, Tamoxifen, Antibody

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The success of antiestrogen therapy for human breast cancer is dependent on close regulation of breast cell growth by hormones. Estrogens promote growth by specific binding to breast cell receptors which, in turn, act as potent nuclear transcription factors (see Fig.1). However, as cancer progresses, receptors for estrogen may be subverted by cross-communication with peptide receptor pathways. HER-2 tyrosine kinase, in combination with HER-3 protein, forms a high affinity receptor for heregulin (HRG), a peptide implicated in the growth control of breast cells. On stimulation, HER-2 receptor promotes signal transduction to the nucleus via specific phosphorylation cascades. Phosphorylation of ER on tyrosine and serine residues is associated with changes in the interaction of ER with DNA and offers a potential link to HER-2 pathways (Fig.1). Since overexpression of HER-2 receptor in breast cancer predicts a poor response to endocrine therapy, understanding the relationship between HER-2 and ER receptors may facilitate patient management and the development of more effective therapies.

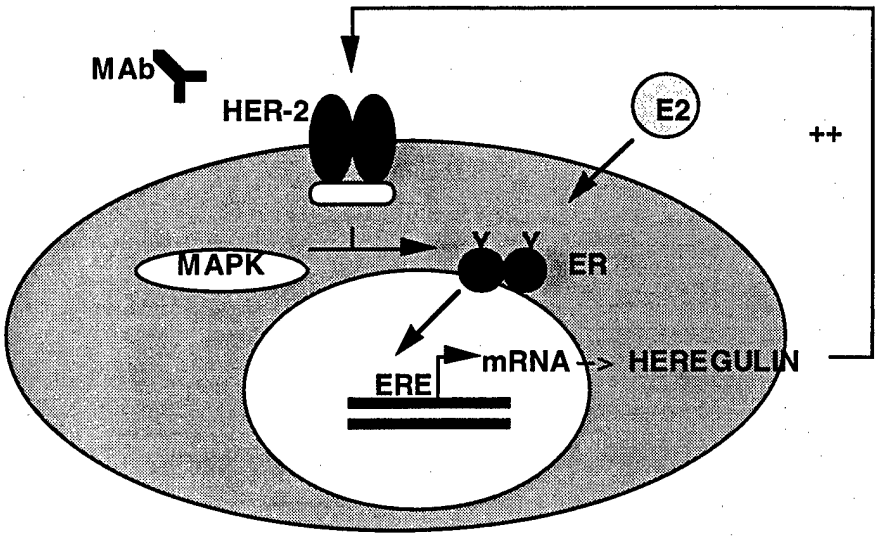

Fig.1. Pathways of estrogen (E2)-dependent and -independent activation of estrogen receptor (ER) in breast cancer cells with HER-2 receptor (HER-2) overexpression. E2 binds ER and promotes receptor dimerization. ER dimers activate estrogen-response elements (ERE) in nucleus and specific transcription. Heregulin may stimulate HER-2 receptor and promote estrogen-independent ER activation (Oncogene 1995; 10: 2435).

We postulate that regulation of ER by HER-2 fosters genesis of estrogen-independent growth. Using estrogen-responsive, human MCF-7 breast cancer cells with low levels of HER-2 gene and bioengineered MCF-7 cells with overexpression of HER-2, we tested growth regulation by estrogen and antiestrogens. Although estradiol elicits increased growth of MCF-7 parent cells ($P<0.001$), the hormone at 5 nM has no effect on the proliferation of MCF-7/HER-2 cells. Treatment of MCF-7 parent cells with the antiestrogen, tamoxifen, leads to a dose-dependent reduction in cell proliferation ($P<0.01$), but MCF-7/HER-2 cells are not affected by tamoxifen. Thus, overexpression of HER-2 gene in estrogen-sensitive MCF-7 cells appears to elicit resistance to endocrine therapy in vitro. Using a nude mouse model, MCF-7

parent cells fail to grow in the absence of estrogen, and, as expected, estradiol promotes an increase in the growth of MCF-7 tumors in vivo. It is notable that treatment with HRG maintains the growth of these estrogen-dependent parental cells in ovariectomized mice even in the absence of estrogen. In vivo, MCF-7 parent cells are sensitive to tamoxifen treatment, but MCF-7/HER-2 cells are unaffected by the drug. Collectively, these findings suggest that, as in the clinic, activation of HER-2 receptors associates with the progression of human breast cancers to a hormone-independent state.

To assess cross-talk between ER and HER-2, we tested whether ER is a substrate for phosphorylation by HER-2 tyrosine kinase. MCF-7 cells were treated with HRG in the absence of estrogen and showed a prominent increase in tyrosine phosphorylation of ER protein, with phosphorylation of ER as early as 1-2 min after HRG. In MCF-7/HER-2 cells, HRG elicits a similar increase in tyrosine phosphorylation of ER, with maximal effects at 5-15 min. This regulation of ER phosphorylation by the HER-2 /HRG pathway suggests that molecular activation of ER may not depend exclusively on estrogen binding (Fig.1). Indeed, in the absence of estrogen, treatment with HRG activates transcription from an ERE-CAT reporter gene transfected in MCF-7 parent cells, suggesting that HRG signaling promotes estrogen-independent activity by ER.

Treatment of MCF-7 cells with estrogen provokes a delayed down-regulation of ER transcripts and protein levels, an autoregulatory circuit serving to limit estrogen action. Analyses of RNA and protein from MCF-7 parent and MCF-7/HER-2 cells show a similar reduction in both ER transcripts (6.5 kb) and [^{3}H]-estradiol-binding activity in breast cells that overexpress HER-2 gene as compared to parent control cells.

Although overexpression of HER-2 gene in MCF-7 tumor cells elicits estrogen-independent growth that is resistant to tamoxifen, MCF-7/HER-2 cells retain sensitivity to a pure antiestrogen, ICI 182.780. In addition, therapy of MCF-7/HER-2 cells with a combination of anti-HER-2 receptor antibody (MAb; Fig.1) and tamoxifen appears to enhance antitumor activity ($P<0.001$). Results of this work will help to guide efforts for development of improved antihormone therapeutics for use in the suppression and prevention of breast cancers with overexpression of HER-2 receptors.

References

1. Pietras RJ, Fendly BM, Chazin V, Pegram MD, Howell SB, Slamon DJ. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene 1994; 9 : 1829-38.

2. Pietras RJ, Arboleda J, Wongvipat PN, Ramos L, Sliwkowski MX, Slamon DJ. HER-2/neu signaling regulates estrogen receptor in breast cancer. Proc Am Assoc Cancer Res 1995; 36 : 254.

3. Pietras R J, Arboleda J, Reese D, Wongvipat PN, Pegram MD, Ramos L, Gorman CM, Parker MG, Sliwkowski MX, Slamon DJ. HER-2 tyrosine kinase pathway

targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 1995; 10 : 2435-46.

4. Pegram MD, Finn RS, Beryt M, Arzoo K, Pietras RJ, Slamon DJ. The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene 1997 (paper in press).

5. Pietras RJ, Pegram MD. Oncogene activation and breast cancer progression. In: Manni A, Conn PM, eds. Contemporary Endocrinology: Endocrinology of the Breast. Humana Press, Inc, 1997 (paper in press).

INHIBITORY EFFECTS OF COMBINATIONS OF HER-2/*NEU* ANTIBODY AND CHEMOTHERAPEUTIC AGENTS USED FOR TREATMENT OF HUMAN BREAST CANCERS

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Running Title: Anti-HER-2/*neu* Antibody and Chemotherapy Combinations

Key Words: HER-2/*neu* (c-*erb*B-2), chemotherapy, breast cancer, multiple drug effects analysis, synergy

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Abstract

Previous studies have demonstrated a synergistic interaction between rhuMAb HER2 and the cytotoxic drug cisplatin in human breast and ovarian cancer cells. To characterize the nature of the interaction between rhuMAb HER2 and other classes of cytotoxic drugs, we used the multiple drug effect analysis method to determine combination index (CI) values for a variety of chemotherapeutic agent/rhuMAb HER2 combinations *in vitro*. SK-BR-3 human breast cancer cells with HER-2/*neu* amplification/overexpression served as the target cell line for *in vitro* cytotoxicity experiments. Synergistic interactions were observed for rhuMAb HER2 in combination with cisplatin (CI=0.48, P=0.003), thiotepa (CI=0.67, P=0.0008), and etoposide (CI=0.54, P=0.0003). Additive cytotoxic effects were observed with rhuMAb HER2 plus doxorubicin (CI=1.16, P=0.13), paclitaxel (CI=0.91, P=0.21), methotrexate (CI=1.15, P=0.28), and vinblastine (CI=1.09, P=0.26). One drug, 5-fluorouracil, was found to be antagonistic with rhuMAb HER2 *in vitro* (CI=2.87, P=0.0001). *In vivo* therapeutic studies were conducted with HER-2/*neu*-transfected, MCF7 human breast cancer cells which, in contrast to SK-BR-3 cells, are tumorigenic in athymic mice. Combinations of rhuMAb HER2 plus cyclophosphamide, doxorubicin, paclitaxel, methotrexate, etoposide, and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone controls ($p<0.05$). Xenografts treated with rhuMAb HER2 plus 5-fluorouracil were not significantly different from 5-fluorouracil alone controls consistent with the subadditive effects observed in the *in vitro* studies. The additive or synergistic therapeutic interaction of rhuMAb HER2 with alkylating agents, platinum analogs, taxanes, anthracyclines, topoisomerase II inhibitors, and some antimetabolites in HER-2/*neu*-overexpressing human breast cancer cells suggests that these are rational combinations to take to human clinical trials.

Introduction

Overexpression of $p185^{HER\text{-}2/neu}$, resulting from amplification of the HER-2/*neu* gene, is associated with poor clinical outcome in 25-30% of carcinomas of the breast (Slamon, *et al.*, 1987), as well as in other human malignancies (Semba, *et al.*,1985; Slamon, *et al.*, 1989; Berchuck, *et al.*, 1991; Yonemura, *et al.*, 1991; Hetzel, *et al.*, 1992; Lukes, *et al.*,1994; Press, *et al.*, 1994; Saffari, *et al.*, 1995). The murine monoclonal antibody 4D5 has specificity for a juxtamembrane epitope in the extracellular domain (ECD) of the $p185^{HER\text{-}2/neu}$ protein (Fendly, *et al.*, 1990) and is capable of eliciting an antiproliferative effect against murine cells transformed by HER-2/*neu* as well as human malignant cell lines and xenografts overexpressing this oncogene (Chazin, *et al.*, 1992). Importantly, this growth inhibitory effect is specific for cells with HER-2/*neu* overexpression and does not occur with cells expressing normal amounts of the protein (Hudziak, *et al.*, 1989; Chazin, *et al.*, 1992). A recombinant, "humanized" form of 4D5 (rhuMAb HER2) has been generated by inserting the complementary-determining regions (CDRs) of 4D5 into the framework of a consensus human IgG_1 (Carter, *et al.*, 1992). When compared to murine 4D5, rhuMAb HER2 exhibits a stronger binding affinity for $p185^{HER\text{-}2/neu}$ but has similar specific antiproliferative activity against HER-2/*neu*-overexpressing cell lines and xenografts.

To determine how best to use this antibody both alone and in combination with established therapeutic agents we undertook a series of studies to evaluate its inhibitory effects in both *in vitro* and *in vivo* preclinical models. These studies

were based on the previously published report of enhanced activity of cisplatin (CDDP) when used in combination with antibodies directed against the epidermal growth factor receptor (EGFR) (Aboud-Pirak, *et al.*, 1988). Initial studies showed that when used in combination with the drug CDDP, 4D5, rhuMAb HER2, as well as other anti-HER-2/*neu* antibodies, potentiate cytotoxicity of the chemotherapeutic by decreasing DNA repair activity following CDDP-induced DNA damage (Hancock, *et al.*, 1991; Pietras, *et al.*, 1994). This effect, termed receptor enhanced chemosensitivity (REC), specifically targets HER-2/*neu*-overexpressing cells and has no effect on cells or tissues expressing physiologic levels of the gene. This previously reported interaction between 4D5 and CDDP in inhibiting HER-2/*neu*-overexpressing cell lines has been shown to be synergistic resulting in a two-log increase in CDDP-induced cytotoxicity as well as pathologic complete remissions in experimental animals bearing HER-2/*neu*-overexpressing human breast cancer xenografts (Pietras, *et al.*, 1994).

Synergy is defined as a combination of two or more drugs which achieve a therapeutic effect greater than that expected by simply adding the effects of the component drugs. Such a synergistic interaction between drugs may improve therapeutic results in cancer treatment if the synergism is specific for tumor cells (Chou, *et al.*, 1984). Moreover, analysis of the nature of the interaction between two drugs (synergism, addition, or antagonism) may yield insight into the biochemical mechanisms of interaction of the drugs. For example, two drugs targeting the same enzyme or biochemical pathway may compete with one another resulting in an antagonistic interaction, whereas two drugs targeting

completely independent pathways may be additive, and one drug which potentiates the action of another may result in therapeutic synergy.

In order to characterize the effects of combinations of rhuMAb HER2 and a series of cytotoxic chemotherapeutic drugs commonly used in breast cancer therapy, we utilized the median-effect approach and combination-index isobologram method of multiple drug effect analysis. Assays were performed for the various drug/rhuMAb HER2 combinations *in vitro* using a cytotoxicity endpoint employing SK-BR-3 human breast cancer cells which contain HER-2/*neu* amplification/overexpression. The median effect principle/combination index isobologram method used to test multiple drug effects allows quantitation of combination index (CI) values at different dose-effect levels based on parameters derived from median-effect plots of the chemotherapeutic drug alone, rhuMAb HER2 alone, and the combination of the two at fixed molar ratios. In this model CI values <1 indicate synergy, CI=1 indicates addition, and CI>1 denotes antagonism (Chou, *et al.*, 1984). We performed this analysis with rhuMAb HER2 in combination with eight drugs representing seven different classes of cytotoxic chemotherapeutics *in vitro*. In addition, to circumvent the possibility that any observed therapeutic effect might be unique to an individual cell line or to a specific method of analysis, parallel studies were conducted *in vivo* with the same rhuMAb HER2/drug combinations. The *in vivo* studies were conducted using an athymic mouse model with HER-2/*neu*-transfected MCF7 human breast carcinoma xenografts which, in contrast to SK-BR-3 cells, are tumorigenic. Using this model we also investigated the effect of various

chemotherapeutic drugs on the pharmacokinetics of rhuMAb HER2 in a subset of mice receiving either rhuMAb HER2 alone or rhuMAb HER-2 plus cytotoxic drug.

Results

Multiple Drug Effect Analysis of rhuMAb HER2 in combination with cytotoxic chemotherapy drugs on SK-BR-3 breast carcinoma cells in vitro

We have previously reported a synergistic interaction between the anti-HER-2/*neu* antibody 4D5 and CDDP resulting in a two log increase in anti-tumor efficacy in treatment of HER-2/*neu* overexpressing human breast and ovarian carcinoma cells and xenografts (Pietras, *et al.*, 1994), and other studies confirm this interpretation (Hancock, *et al.*, 1991; Arteaga, *et al.*, 1994). Subsequent to this report, the anti-HER-2/*neu* 4D5 antibody was humanized so that it would be suitable for repeated dosing in human subjects without the induction of a human anti-mouse antibodies which have proven to be a limitation for the therapeutic use of other murine monoclonal antibodies (Carter, *et al.*, 1992). To extend the observations made with murine monoclonal antibody 4D5 in combination with CDDP and to conduct a comprehensive survey of rhuMAb HER2 used in combination with other classes of cytotoxic chemotherapeutic drugs available for clinical use, rhuMAb HER2 was analyzed in combination with cytotoxic chemotherapeutic drugs representing seven different drug classes. The drugs studied include an anthracycline antibiotic, doxorubicin (DOX), a taxane,

paclitaxel (TAX), the topoisomerase II inhibitor etoposide (VP-16), a platinum analog cisplatin (CDDP), a vinca alkaloid vinblastine (VBL), alkylating agents, thiotepa (TSPA) for *in vitro* experiments and cyclophosphamide (CPA) for *in vivo* experiments, and the antimetabolites methotrexate (MTX) and 5-fluorouracil (5-FU).

In this analysis, dose response curves were constructed for each drug alone, rhuMAb HER2 alone, and the combination at fixed molar ratios. In order to insure the accuracy and reproducibility of the *in vitro* experiments, seeding density of the target SK-BR-3 cells in each plate were carefully controlled such that absorbency values for the control wells were not statistically significantly different from plate to plate, allowing for more precise comparisons between repeated experiments. A representative experiment of the multiple drug effect analyses performed for all chemotherapeutic agents in combination with rhuMAb HER2 is shown using the alkylating agent thiotepa (Figure 1, A and B, Tables 1 and 2). In this analysis Fa and Fu are the fractions of SK-BR-3 cells affected or unaffected, respectively, by the dose (D) of either agent (drug or antibody). Dm is the dose required to produce the median effect (analogous to the IC_{50}), and m is the Hill coefficient used to determine whether or not the dose effect relationships follow sigmoidal dose-response curves (Hill, 1913). The linear regression correlation coefficients, (r-values) of the median effect plot demonstrate the validity of this methodology (Table 2) (Chou, *et al.,* 1984). CI values for the combination of TSPA and rhuMAb HER2 are significantly less than 1.0 across all combined doses tested indicating a synergistic interaction (Fig. 1B). A summary

of the data from the same analysis applied to each of the drugs tested demonstrates that CDDP, TSPA, and VP-16 exhibit synergistic therapeutic interactions (CI<1; P<0.05) with rhuMAb HER2 in SK-BR-3 human breast carcinoma cells across a wide range (~0.2 - 0.8) of Fa values (Table 3). Additive interactions (CI=1) were observed for TAX, DOX, MTX, and VBL in combination with rhuMAb HER2, while only one drug, 5-FU, was found to exhibit an antagonistic (CI>1; p<0.05) interaction (Table 3).

$P185^{HER\text{-}2/neu}$ expression and tyrosine phosphorylation following exposure to cytotoxic agents

Other investigators have demonstrated that exposure of several cancer cell lines to the anthracycline DOX results in an increase in expression of the EGFR and/or its ligand TGF-α (Zuckier and Tritton, 1983; Hanauske, *et al.*, 1987; Baselga, *et al.*, 1992; Baselga, *et al.*, 1993), and this phenomenon has been proposed to explain the synergistic cytotoxic effects of DOX used in combination with anti-EGFR monoclonal antibodies. To test whether $p185^{HER\text{-}2/neu}$ expression is similarly altered by DOX, protein expression levels were measured following exposure to this drug (Figure 2). These studies demonstrate that, following exposure to DOX, $p185^{HER\text{-}2/neu}$ expression levels in SK-BR-3 breast carcinoma cells are unaltered unlike the effects on EGFR expression in A431 cells (Baselga, *et al.*, 1992). To extend these studies, we explored the possibility that cytotoxic drugs may impact $p185^{HER\text{-}2/neu}$ functional activity rather than expression levels by testing the effect of the various cytotoxic drugs on the ability of 4D5 to

induce tyrosine phosphorylation of $p185^{HER\text{-}2/neu}$ (Yarden, 1990). SK-BR-3 cells were treated with cytotoxic drugs, then allowed to incubate with 4D5 (12.5ug/ml) or control murine monoclonal IgG for 5 min. at 37°C. Protein lysates were then quantitated and analyzed by anti-phosphotyrosine immunoblot. These studies demonstrate an increase in $p185^{HER\text{-}2/neu}$ tyrosine phosphorylation following incubation with 4D5 compared to a non-specific isotype control antibody (Figure 3, lanes 1 and 2). Prior exposure of the cells to the 3 drugs which were found to be synergistic with anti-HER-2/*neu* antibody (CDDP, TSPA, and VP-16) had no effect on 4D5-induced p185 tyrosine phosphorylation (Figure 3, lanes 3-7 and lanes 9 and 10). Similarly, neither DOX which is additive, nor 5-FU which is antagonistic, had effects on p185 tyrosine phosphorylation (Figure 3, lanes 8 and 11). Taken together these data demonstrate that none of the synergistic, additive, or antagonistic effects of chemotherapeutic drugs with anti-HER-2/*neu* antibody are explained on the basis of either chemotherapy-induced alteration of $p185^{HER\text{-}2/neu}$ protein expression levels or its phosphorylation.

Anti-HER-2/neu antibodies alter cell cycle distribution of HER-2/neu-overexpressing human breast cancer cells

The cytotoxic effects of most antimetabolite drugs are presumed to be cell cycle dependent (Tannock, 1978). To identify a possible mechanism for the antagonism of 5-FU with rhuMAb HER2 we investigated the effects of murine 4D5 and rhuMAb HER2 on cell cycle distribution of exponentially growing SK-BR-3 and MCF7 cells *in vitro* (Figures 4 and 5). Both the murine 4D5 and

rhuMAb HER2 antibodies reduce the percentage of cells undergoing S-phase as well as increase the percentage of cells in G0/G1, and these effects are dose-dependent with the maximal antiproliferative activity occurring at antibody concentrations between 1 and 10 ug/ml (Figure 5). There was no significant difference in the magnitude of decrease in S phase fraction of SK-BR-3 cells comparing 4D5 and rhuMAb HER2 indicating the humanization of the murine antibody did not adversely impact its antiproliferative activity. The lack of any effect on cell cycle distribution of non-HER-2 overexpressing MCF7 cells demonstrates the specificity of these antibodies for cells with HER-2/*neu* overexpression. These data are consistent with the hypothesis that a rhuMAb HER2 - induced decrease in the percentage of SK-BR-3 cells in S-phase is associated with a decrease in the cytotoxicity of 5-FU. An antagonistic interaction for the combination of rhuMAb HER2 with another antimetabolite, MTX, was not observed*in vitro*. This apparent lack of antagonism between MTX and rhuMAb HER2 *in vitro* may be due to the longer incubation period required for MTX (120 vs. 72 hours) to elicit cytotoxicity in the assay used for the multiple drug effect analysis, and the fact that MTX exerts cytotoxic effects in other phases of the cell cycle (Buick, 1994).

Effect of rhuMAb HER2 in combination with multiple chemotherapeutic drugs on growth of HER-2/neu-transfected MCF7 breast xenografts in vivo

To further evaluate the potential therapeutic effects of rhuMab HER2/chemotherapy combinations and to extend our observations beyond a

single cell line and preclinical model, a series of *in vivo* studies of chemotherapy with rhuMAb HER2 against human breast cancer xenografts in athymic mice were performed. All of the doses, routes of administration, and dose intervals for the various cytotoxic drugs and rhuMAb HER2 were based on independent dose finding experiments for this specific strain, age, weight, and sex of athymic mouse. The cytotoxic drug doses used were at or near the maximum tolerated doses previously reported in the literature (Giovanella, *et al.*, 1977; Boven and Winograd, 1991).

For the alkylating agent cyclophosphamide CPA, combination with rhuMAb HER2 resulted in a significant reduction ($P<0.05$) in day 21 xenograft volume compared to either agent alone (Figure 6A). The combination of the anthracycline antibiotic DOX plus rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to either single agent alone (Figure 6B). The combination of the taxane compound TAX plus rhuMAb HER2, which demonstrated an additive interaction *in vitro*, resulted in a significant reduction in day 20 xenograft volume compared to treatment with TAX alone (Figure 6C). However, the difference between rhuMAb HER2 alone and rhuMAb HER2 plus TAX did not reach statistical significance. This is likely due to the relatively small sample size in each group and the fact that the dose of rhuMAb HER2 in this particular analysis (10mg/kg IP twice weekly) yielded a marked reduction in xenograft growth even when used as a single agent.

The following four drug/rhuMAb HER2 combinations were studied in a single large experiment. For this experiment, a "rational dose" (RD) of rhuMAb

HER2 was chosen as new information became available based on comparative pharmacokinetic studies from both humans and athymic mice. RD is the dose of a given drug which can reproduce a serum level in nude mice similar to that observed in human subjects (Inaba, *et al.,* 1988). The RD for rhuMAb HER2 resulted in a lower cumulative rhuMAb HER2 dose (16mg/kg vs. 30-50mg/kg) during the 21 day observation period for this experiment compared to the three*in vivo* studies reported above. With this approach, a significant reduction in day 21 xenograft volume was observed for the topoisomerase II inhibitor VP-16 when used in combination with rhuMAb HER2 compared to either agent alone (Figure 7A). The combination of the microtubule inhibitor VBL with rhuMAb HER2 also significantly reduced MCF7/HER-2 xenograft volume compared to treatment with VBL alone or single agent rhuMAb HER2 (Figure 7B). For the antimetabolite class of cytotoxic chemotherapeutics, two drugs with clinical activity against breast cancer were chosen for combination studies. Treatment with MTX, which targets dihydrofolate reductase, in combination with rhuMAb HER2 resulted in a significant reduction in day 21 MCF7/HER-2 xenograft volume when compared to either MTX alone or rhuMAb HER2 alone (Figure 7C). However, the antimetabolite drug 5-FU, which targets thymidylate synthetase, and which was found to be antagonistic when combined with rhuMAb HER2 *in vitro*, did not yield a significant reduction in xenograft volume when compared to 5-FU alone *in vivo* (Figure 7D). Although the combination of rhuMAb HER2 plus 5-FU was superior to rhuMAb HER2 alone in this experiment ($P<0.05$), the 5-FU dose used had sufficient efficacy as a single agent such that it was not possible to resolve

potential differences between 5-FU alone and the combination with the sample sizes chosen (10 mice/group).

Correlation between rhuMAb HER-2 serum concentration and MCF7/HER-2 xenograft volume

To investigate the relationship between rhuMAb HER2 concentration and xenograft size, trough rhuMAb HER2 serum concentration was measured in a subset of mice on day 64 following extended rhuMAb HER2 treatment at the RD (8mg/kg loading dose and eight weekly I.P. injections of 4mg/kg) (Figure 8). A significant inverse correlation (Spearman Rank Correlation rho = -0.543; P = 0.0067) between trough rhuMAb HER2 concentration and xenograft volume was observed, suggesting that the MCF7/HER-2 xenograft size significantly affects rhuMAb HER2 pharmacology. Furthermore, this effect is independent of serum shed HER-2/*neu* ECD concentration as this molecule was undetectable in any of the murine serum samples analyzed (data not shown).

To determine if chemotherapeutic drugs have an effect on rhuMAb HER2 pharmacology, day 64 trough serum rhuMAb HER2 concentrations were analyzed by treatment group in a subset of mice used for the *in vivo* studies. Controlling for xenograft size, there was no significant difference in rhuMAb HER2 trough concentration between any of the treatment groups in figure 8 (data not shown).

Discussion

The protein products of transforming oncogenes have been a target for anticancer drug development since the initial discovery of these genes, yet there are currently no drugs specifically targeting these proteins available for clinical use. Identification of the HER-2/*neu* alteration and its association with aggressive forms of human breast cancer has resulted in its successful therapeutic targeting (Slamon, *et al.*,1987; Slamon, *et al.*, 1989; Baselga, *et al.*, 19[illegible] The interaction of anti-HER-2/*neu* antibodies with $p185^{HER\text{-}2/neu}$, the gene product of the transforming proto-oncogene HER-2/*neu* (c-*erb*B-2), results in receptor tyrosine phosphorylation (Yarden, 1990), downregulation or receptor expression (Park, *et al.*, 1992), internalization of the antibody-receptor complex (Ma[illegible], *et al.*, 1991), and a decrease in the association of $p185^{HER\text{-}2/neu}$ with its co-receptor HER-3 (Reese, *et al.*, 1996; Klapper, *et al.*, 1997). These events are accompanied by a number of biological effects including a decrease in cell proliferation (Rodriguez, *et al.*, 1993), alteration of cell cycle distribution, and a marked decrease in the ability of the cell to excise and repair DNA damage induced by platinum analogs (Hancock, *et al.*, 1991; Arteaga, *et al.*, 1994; Pietras, *et al.*, 1994). This enhanced cytotoxic activity is specific for malignant cell lines or xenografts with HER-2/*neu* receptor overexpression since anti-HER-2/*neu* antibodies have no such effect on cell lines with physiologic HER-2/*neu* expression levels (Pietras, *et al.*, 1994). Interaction between the $p185^{HER\text{-}2/neu}$ signaling pathway and CDDP-DNA repair mechanisms has been further demonstrated using tyrosine kinase inhibitors to block $p185^{HER\text{-}2/neu}$ receptor

phosphorylation which inhibits antibody induced attenuation of repair of platinum-DNA adducts (Arteaga, *et al.*, 1994). Moreover, reversal of CDDP resistance is possible through transfection and overexpression of HER-2/*neu* cDNA followed by incubation with anti-HER-2/*neu* antibody (Pietras, *et al.*, 1994). As a result of this work, the clinical efficacy of the combination of an anti-HER-2/*neu* antibody plus CDDP has been demonstrated in breast cancer patients with HER-2-overexpressing breast carcinomas who previously exhibited clinical drug resistance to cytotoxic therapy (Pegram, *et al.*, 1995).

To test whether the receptor enhanced chemosensitivity mechanism may be observed with other classes of cytotoxic chemotherapeutic agents, a comprehensive survey of combinations of rhuMAb HER2 with cytotoxic agents representing seven classes of chemotherapeutics in common clinical use was conducted. Data from multiple drug effect analyses are useful, not only in establishing hypotheses as to the mechanism of action of multi-drug combinations, but can also provide insight as to how two drugs should be administered temporally to gain the maximum therapeutic effect. For example, two drugs which are synergistic might best be administered together whereas two antagonistic drugs would be most effective if given sequentially. Data from the current study demonstrate that the platinum compound CDDP, the alkylating agent TSPA, and the topoisomerase II inhibitor VP-16 are synergistic in combination with rhuMAb HER2 in treating HER-2/*neu*-overexpressing SK-BR-3 breast carcinoma cells *in vitro*. These results suggest the possibility of an interaction between the HER-2/*neu* signaling pathway and intracellular DNA

repair mechanisms involved with repair of DNA damage resulting from each of these DNA damaging agents. Other potential mechanisms could also explain the synergy observed between rhuMAb HER2 and these agents, including the possibility that rhuMAb HER2 could impact the cellular pharmacology of these drugs resulting in an increase in their cytotoxic activity. An argument against this hypothesis is the fact that the anti-HER-2/*neu* antibody has no effect on the net cellular incorporation of ^{14}C-labeled carboplatin (Pietras, *et al.*, 1994) or ^{14}C-doxorubicin (Pegram, *et al.*, 1993). Another possible mechanism for synergy with rhuMAb HER2 is an effect of cytotoxic drugs on the expression level and/or kinase activity of p185$^{HER\text{-}2/neu}$. An analogous mechanism has been postulated for the EGFR where low doses of DOX appear to increase EGFR expression which enhances the antiproliferative activity of anti-EGFR antibody (Zuckier and Tritton, 1983; Hanauske, *et* al., 1987; Baselga, *et al.*, 1992; Baselga, *et al.*, 1993). Experiments designed to evaluate this possibility with anti-HER-2/*neu* antibody failed to demonstrate any change in p185$^{HER\text{-}2/neu}$ expression or 4D5-induced receptor tyrosine phosphorylation following exposure to cytotoxic drugs, suggesting that this mechanism is not operative for the HER-2/*neu* receptor.

Many of the rhuMAb HER2/drug combinations tested in this study demonstrate additive interactions suggesting that the observed antiproliferative effects of rhuMAb HER2 plus drug are due to the action of each agent acting independently. It is interesting to note that the mechanism of action of many of these drugs does not involve direct DNA damage, but rather involves disruption of microtubule polymerization/depolymerization (taxanes and vinca alkaloids) or

inhibition of DNA synthesis (antimetabolites). This observation is consistent with the hypothesis that synergy between cytotoxic drugs and rhuMAb HER2 involves an interaction between the HER-2/*neu* signaling and DNA repair pathways. The antimetabolite drug 5-FU was the only drug which demonstrated antagonism when used in combination with rhuMAb HER2 *in vitro*. We have not yet defined the exact mechanism for this antagonism, but it may be the result of the alteration of cell cycle distribution caused by rhuMAb HER2. It could also be the result of intracellular pharmacological effects, alteration of the enzymatic activity responsible for conversion of 5-FU to 5-fluorodeoxyuridine monophosphate, or an impact on the level of the target enzyme thymidylate synthetase. Further work is needed to explore these possibilities.

The multiple drug effects model is not easily applicable to the analysis of *in vivo* studies since such an analysis, using the number of drugs reported in this study, would require at least 600 athymic mice (assuming 5 mice per group, 5 data points for each dose response curve, and 3 dose response curves - for each drug alone, and the combination). As a result we used a more conventional approach for analysis of the *in vivo* data (i.e. single factor ANOVA at a fixed time point following treatment of mice with optimal drug or rhuMAb HER2 doses). The cytotoxic drug doses chosen for these experiments were based on independent dose-escalation studies and are at or near the MTD reported in the literature for each of these drugs. The rhuMAb HER2 doses and schedules for these experiments were designed to achieve target serum concentrations of $\geq$ 10 - 20 ug/ml in mice bearing HER-2/*neu*-overexpressing xenografts of 50 - 500 mm^3.

This antibody concentration is associated with maximal antiproliferative effects *in vitro* (De Santes, *et al.*, 1992). We were able to demonstrate statistically superior anti-tumor efficacy of rhuMAb HER2 in combination with TAX, CPA, MTX, VP-16, DOX, and VBL compared to chemotherapeutic drug alone. These results are consistent with the *in vitro* data which demonstrate that rhuMAb HER2 is either additive or synergistic with each of these drugs. For the drug 5-FU, which was antagonistic with rhuMAb HER2 *in vitro*, the combination *in vivo* was superior to rhuMAb HER2 alone but not to 5-FU alone. Although this could be secondary to an antagonistic effect, it is also possible that the sample sizes in each treatment group were not sufficient to discriminate between 5-FU alone and the combination, especially in light of the fact that 5-FU given as a single agent had a marked effect on xenograft volume in this model.

There are additional reasons why the *in vitro* data cannot be compared directly to the *in vivo* data. The cell lines used for the *in vitro* and *in vivo* studies were different since SK-BR-3 cells are not tumorigenic and therefore can not be used for *in vivo* studies. The monolayer cell proliferation assay tests short-term, constant drug exposure, whereas the *in vivo* assays involved repeated drug exposures over longer time periods. The pharmacology of the agents tested *in vivo* is vastly different from the *in vitro* model due to phenomena such as drug metabolism and regional differences in drug pharmacokinetics within xenografts due to differences in tumor blood flow, oncotic pressures, pH, and pO_2. Despite these differences, the *in vivo* model demonstrates enhanced efficacy of rhuMAb HER2/cytotoxic drug combinations compared to single agents alone for most of

the drugs tested. In addition there was no evidence that rhuMAb HER2 had any deleterious effect on chemotherapeutic drug efficacy *in vivo*. Additionally, we did not observe any overt increase in toxicity, as determined by measurement of animal weights, observation of activity level, or overall survival, in mice treated with rhuMAb HER2/chemotherapy combinations.

Previous analysis of rhuMAb HER2 pharmacokinetics in human subjects demonstrated an inverse association between serum concentrations of rhuMAb HER2 and the shed HER-2/*neu* ECD (Baselga, *et al.*, 1996). One mechanism which might explain this observation is the direct binding of rhuMAb HER2 to shed HER-2/*neu* ECD in the circulation resulting in a more rapid clearance of the resulting antigen/antibody complex by the reticuloendothelial system. Another potential mechanism is that high serum shed HER-2/*neu* ECD may be a marker of increased tumor burden, and therefore the inverse association between rhuMAb HER2 concentration and shed HER-2/*neu* ECD may be due to increased binding and turnover of rhuMAb HER2 directly by tumor cells. In the MCF7/HER-2 xenograft model, we measured rhuMAb HER2 trough concentration, shed HER-2/*neu* ECD, and tumor volume concurrently. These data demonstrate a statistically significant inverse relationship between rhuMAb HER2 trough concentration and xenograft volume. This relationship is independent of serum shed HER-2/*neu* ECD since no serum shed HER-2/*neu* ECD could be detected using a sensitive ELISA assay (Sias, *et al.*, 1990) in this model. These data demonstrate that shed HER-2/*neu* ECD is not required for HER-2/*neu* - overexpressing tumors to affect rhuMAb HER2 pharmacokinetics.

Prior treatment with the drugs MTX, 5-FU, VP-16, and VBL *in vivo* had no effect on rhuMAb HER2 trough levels in murine serum. Moreover, concomitant administration of the drug CDDP had no impact on mean pharmacokinetic parameters of rhuMAb HER2 in a phase II clinical trial of CDDP plus rhuMAb HER2 in 39 patients with advanced breast cancer (Pegram, *et al.*, 1995). Taken together, these data suggest that cytotoxic chemotherapeutics drugs have no effect rhuMAb HER2 pharmacokinetics *in vivo.*

It has been widely accepted that identification of molecular alterations which play a role in the pathogenesis of human malignancies will lead to the development of rational targeted therapeutics which hopefully will be more effective and less toxic than those currently available in clinical practice. The HER-2/*neu* alteration in human breast cancer is the first of what is hoped to be a number of molecular targets for future drug design for this disease as well as other human cancers. Studies leading to a greater understanding of the effects of HER-2/*neu* therapy in combination with traditional therapeutics should lead to the development of clinical trials which demonstrate the optimal utility of this molecularly-targeted approach. The additive or synergistic therapeutic interaction of rhuMAb HER2 with most chemotherapeutic drugs suggests that these are rational combinations to take into human clinical trials.

Materials and Methods

Multiple Drug Effect Analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic agents against HER-2/neu-overexpressing SK-BR-3 breast carcinoma cells in vitro

Aliquots of 5 X 10^3 SK-BR-3 cells were plated in 96-well microdilution plates. Following cell adherence (24h), experimental media containing either rhuMAb HER2 (Genentech, Inc. South San Francisco, CA) or control media was added to appropriate wells. After incubation for 24h, chemotherapeutic agent or control solution was added to triplicate wells and serial two-fold dilutions were performed to span the dose range suitable for the dose-effect analysis for rhuMAb HER2 and each of the cytotoxic drugs. The dose ranges tested for rhuMAb HER2 and each drug tested in these experiments are listed in Table 3. Eight cytotoxic drugs representative of seven different classes of cytotoxic chemotherapeutic agents were analyzed including: platinum analogs - cisplatin (CDDP; Bristol Laboratories, Princeton, NJ); anthracycline antibiotics - doxorubicin (DOX; Cetus Corporation, Emeryville, CA); alkylating agents - thiotepa (TSPA; Lederle Laboratories, Pearl River, NY); taxanes - paclitaxel (TAX; Mead Johnson, Princeton, NJ); vinca alkaloids - vinblastine (VBL; Eli Lilly Co., Indianapolis, IN); topoisomerase II inhibitors - etoposide (VP-16; Bristol Laboratories, Princeton, NJ); and antimetabolites - 5-fluorouracil (5-FU; solo Park Laboratories, Inc., Elk Grove Village, IL) and methotrexate (MTX; Immunex Corporation, Seattle, WA).

Following incubation for 72h (120h for MTX) plates were washed with PBS and stained with 0.5% N-Hexamethylpararosaniline (crystal violet) in methanol. Sorenson's buffer (0.025M sodium citrate, 0.025M citric acid in 50% ethanol) 0.1 ml was added to each well, and the plates were analyzed in an ELISA plate reader at 540 nm wavelength. Absorbance at this wavelength correlates with cell survival (Flick and Gifford, 1984; Gillies, *et al.*, 1986; Reile, *et al.*, 1990). Absorbance values from control wells in each plate were compared statistically to ensure even loading of cells from plate to plate for each experiment. Multiple drug effect analysis was performed using computer software (Biosoft, Cambridge, England). Combination index (CI) values were derived from parameters of the median effect plots and statistical tests were applied (student t-test) to determine if the mean CI values resulting from separate experiments at multiple effect levels were significantly different from CI=1.

Western blot analysis

SK-BR-3 cells were allowed to incubate with cytotoxic drugs at the IC_{30} concentration for the times indicated in figures 2 and 3. Cells were then washed in PBS and lysed at 4°C in 20mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 5mM EDTA, 1mM sodium orthovanadate, 1 mM phenylmethyl-sulfonylfluoride, leupeptin 1ug/ml and aprotinin 1ug/ml. Insoluble material was cleared by centrifugation and protein was quantitated using BCA (Pierce Biochemicals, Rockford, IL), resolved by SDS-PAGE, and transferred to immobilon-P (Millipore,

Bedford, MA). $P185^{HER\text{-}2/neu}$ was detected using anti-c-*neu* (Oncogene Science, Uniondale, NY) and anti-phosphotyrosine immunoblotting was performed using monoclonal antibody PY20 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell cycle analysis

SK-BR-3 or MCF7 breast cancer cells were plated at a density of 2 X 10^6/dish in 60 X 15-mm culture dishes and allowed to adhere overnight. Monolayers were washed with PBS and allowed to incubate with media containing anti-HER-2 or control antibodies at concentrations of 0.01 - 10ug/ml. Following 72h incubation, cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at -20°C. Fixed cells were then washed twice with PBS and allowed to incubate with RNAse 100ug/ ml (Worthington Biochemical) for 30 min at 37°C. Following centrifugation, nuclei were subjected to propidium iodide 50ug/ml (Molecular Probes, Inc.) staining in PBS. Samples were analyzed by flow cytometry (Epics Elite, Coulter Corporation) using Modfit LT software (Verity Software House).

Analysis of rhuMAb HER2 in combination with cytotoxic chemotherapeutic drugs against HER-2/neu-overexpressing breast carcinoma xenografts in vivo

HER-2/*neu*-transfected MCF7 cells which express high levels of $p185^{HER\text{-}2/neu}$ and form xenografts in athymic mice were injected subcutaneously (S.Q.) at ~1.0 X 10^7 cells/tumor in the mid-back region of 4-6 week old, female, CD-1

(*nu/nu*), athymic mice (Charles River Laboratories, Wilmington, MA). Prior to cell injection, all mice were primed with 17ß-estradiol (Innovative Research of America, Sarasota, FL) applied S.Q. (1.7mg estradiol/pellet) to promote tumor growth. Tumor volumes, calculated as the product of length, width, and depth, were monitored twice weekly by serial micrometer measurements by a single observer. Five to ten animals were randomly assigned to each treatment group. Statistical tests were performed (single-factor ANOVA) to assure uniformity in starting tumor volumes between treatment and control groups at the beginning of each experiment. All drugs, with the exception of VP-16 which was administered S.Q., were administered by intraperitoneal (I.P.) injection. The dosage of chemotherapeutic agents tested were as follows: DOX (5mg/kg, day 1), MTX (2mg/kg, days 1-5), VP-16 (20mg/kg, days 1-3), 5-FU (16 mg/kg, days 1-4), VBL (0.8mg/kg, days 1 and 2), cyclophosphamide (CPA; 80mg/kg, days 0, 4, 8), and TAX (15mg/kg, days 1-3). These doses were based on independent dose-finding experiments conducted in our laboratory and were near the maximum-tolerated dose for this specific age and strain of female athymic mice. To assure accurate dosing, drug doses were individualized based upon animal weights determined immediately prior to each injection. Treatment with control antibody, cytotoxic drug, rhuMAb HER2, or the combination was initiated 9 to 14 days status post xenograft inoculation at which time xenograft volumes measured ~ 50-100mm^3. Differences in day 21 xenograft volumes between groups were analyzed by single-factor ANOVA of the log transformed tumor volume data. Three dosing schedules of rhuMAb HER2 were used for these experiments. All

dosing schedules were designed to achieve target serum concentrations of ≥10-20 μg/mL during the time chemotherapeutics agents were administered. For the *in vivo* experiments with MTX, VP-16, 5-FU, and VBL, the loading dose of rhuMAb HER2 was 8mg/kg, and the weekly maintenance dose was 4mg/kg. For the experiments with DOX and CPA, the dose of rhuMAb HER2 was 10mg/kg, day 0, 4, and 8. And for the *in vivo* experiment with TAX, the rhuMAb HER2 dose was 10mg/kg twice per week. Human myeloma IgG-1 (Calbiochem-Novabiochem, La Jolla, CA) served as the control antibody for these experiments and was administered at the same dose and dose interval as rhuMAb HER2.

Measurement of rhuMAb HER2 in murine serum

The trough concentration of rhuMAb HER2 in mouse serum was measured using an ELISA with the extracellular domain (ECD) of $p185^{HER\text{-}2/neu}$ as the coat antigen. In this format, 100 μl of $p185^{HER\text{-}2/neu}$ (Genentech, Inc.) was added to MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) at 1 mg/ml in 0.05 M sodium carbonate, pH 9.6. After overnight incubation at 2-8°C, plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween-20) using a Biotek EL304 platewasher (Bio-tek Instruments, Inc., Winooski, VT). Plates were then blocked with 200 μl/well of ELISA diluent (PBS containing 0.5% BSA, 0.05% Tween-20, and 0.05% Proclin300, pH 7.2) for 1-2h at ambient temperature with agitation. After blocking, plates were washed again three times with ELISA wash buffer. Subsequently, 100 μl of standards, samples,

or controls were added to duplicate wells and allowed to incubate for 1h at ambient temperature. After incubation, the plates were washed six times in ELISA wash buffer, and 100 µl of PBS, pH 7.2, containing 2.2 mmol orthophenylene diamine (OPD), (Sigma Chemical Co.) and 0.012% (vol/vol) hydrogen peroxide (H_2O_2; Sigma Chemical Co.) were added to each well. When color had fully developed, the reaction was quenched with 100 µl/well of 4.5 molar sulfuric acid. Absorbence values at 492 nm minus 405 nm reference absorbence were measured using an automatic plate reader (Molecular Devices, Palo Alto, CA). A 4-parameter curve fit program was used to generate the standard curve, from which sample and control concentrations were interpolated (SOFTmax). The standard curve range for the assay was 1.56 to 100 mg/ml.

Detection of p185$^{HER\text{-}2/neu}$ extracellular domain in murine serum

The method for detection of shed HER-2/*neu* extracellular domain (ECD) levels in serum has been described in detail elsewhere (Sias, *et* al., 1990). Briefly, the ELISA employs pairs of anti-HER-2/*neu* monoclonal antibodies (Genentech, Inc.) which recognize mutually exclusive determinants of the extracellular domain of p185 $^{HER\text{-}2/neu}$. Wells were coated overnight at 4ºC with MAb 7F3 which does not compete with rhuMAb HER2 ECD binding. Assay standards (recombinant, p185 $^{HER\text{-}2/neu}$ ECD) and murine serum samples were added to appropriate wells and allowed to incubate for 2h. Following a wash step, secondary antibody was added (MAb 4D5 to detect free shed HER-2 ECD,

and MAb 2C4 to detect total shed HER-2 ECD) for 2h. The bound conjugate is detected with OPD substrate and the resulting absorbence is measured at 490 nm wavelength. The range of the assay is 8.3 to 1800 ng/ml.

REFERENCES

Aboud-Pirak E, Hurwitz E, Pirak ME, Bellot F, Schlessinger J, & Sela M. (1988) J.Natl. Cancer Instit., **80,** 1605-11.

Arteaga CL, Winnier AR, Poirier MC, Lopez-Larraza DM, Shawver LK, Hurd SD, & Stewart SJ. (1994) Cancer Res., **54,** 3758-65.

Baselga J, Miller W, Norton L, et al: (1992) Proc. American Assoc. Cancer Res., **33,** 2947.

Baselga J, Norton L, Masui H, Pandiella A, Coplan K, Miller WH Jr, & Mendelsohn. (1993) J. Natl. Cancer Instit, **85,** 1327-33.

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, et al. (1996) J. Clin. Oncol. **14,** 737-44.

Berchuck A, Rodriguez G, Kinney RB, Soper JT, Dodge RK, Clarke-Pearson DL, & Bast RC Jr. (1991) American J. Obstet. Gynecol, **164,** 15-21.

Boven and Winograd, eds. (1991) The Nude Mouse in Oncol. Res.. CRC Press, Boca Raton, FL.

Buick RN. (1994) in Cancer Chemotherapy Handbook, Dorr & Von Hoff, eds. pp. 3-14, Appleton & Lange, Norwalk, CN.

Carter P, Presta L, Gorman CM, Ridgway JB., Henner D, Wong WL. et al. (1992) Proc. Natl. Acad. Sci. USA, **89,** 4285-9.

Chazin VR, Kaleko M, Miller AD, & Slamon DJ. (1992) Oncogene, **9,** 1859-66.

Chou T. and Talalay P. (1984) Advances in Enzyme Regulation, **22,** 27-55,

De Santes K, Slamon D, Anderson SK, Shepard M, Fendly B, Maneval D, & Press O. (1992) Cancer Res., **52,** 1916-23.

Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, & Ullrich A. (1990) Cancer Res. **50,** 1550-8.

Flick DA,and Gifford GE. (1984) J. Immunol. Methods, **68,** 167-75.

Gillies RJ, Didier N, & Denton M. (1998) Analytical Biochem., **159,** 109-13.

Giovanella, et al., (1977) Proc. Second Internatl. Workshop on Nude Mice, Univ. of Tokyo Press, , pp. 475-481.

Hanauske AR, Osborne CK, Chamness GC, Clark GM, Forseth BJ, Buchok JB, Arteaga CL, & Von Hoff DD. (1987) Euro. J. Cancer & Clin. **.23,** 545-51.

Hancock MC, Langton BC, Chan T, Toy P, Monahan JJ, Mischak RP, & Shawver LK. (1991) Cancer Res. **51,** 4575-80.

Hetzel DJ, Wilson TO, Keeney GL, Roche PC, Cha SS, & Podratz KC. (1992) Gynecol. Oncol., **47,** 179-85.

Hill AV. (1913) Biochem. J. **7,** 471-480.

Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, & Ullrich A. (1989) Molec.& Cell. Biol. **9,** 1165-72.

Inaba M, Kobayashi T, Tashiro T and Sakurai Y. (1988) Jpn. J. Cancer Res. (Gann), **79,** 509.

Kelley RF, O'Connell MP, Carter P, Presta L, Eigenbrot C, Covarrubias M, Snedecor B, Bourell JH, & Vetterlein D. (1992) Biochemistry, **31,** 5434-41.

Klapper LN, Vaisman N, Hurwitz E, et al: (1997) Oncogene **14,** 2099-2109.

Lukes AS, Kohler MF, Pieper CF, Kerns BJ, Bentley R, Rodriguez GC, Soper JT, Clarke-Pearson DL, Bast RC Jr, & Berchuck A. (1994) Cancer, **73,** 2380-5.

Maier LA, Xu FJ, Hester S, Boyer CM, McKenzie S, Bruskin AM, Argon Y, & Bast RC Jr. (1991) Cancer Res. **51,** 5361-9.

Park JW, Stagg R, Lewis GD, Carter P, Maneval D, Slamon DJ, Jaffe H, & Shepard HM. (1992) Cancer Treatment & Res. **61,** 193-211.

Pegram, MD, Pietras RJ, and Slamon DJ. (1992) Proc. Am. Assoc. Cancer Res. **33,** 442.

Pegram, MD, A Lipton A, Pietras R, Hayes D, Weber B, Baselga J, Tripathy D, Twaddell T, Glaspy J, and Slamon. J. (1995) . Proc. Am. Soc. Clin. Oncol. **14** 106.

Pietras RJ, Fendly BM, Chazin VR, Pegram MD, Howell SB, & Slamon DJ. (1994) Oncogene **9,** 1829-38.

Press MF, Pike MC, Hung G, Zhou JY, Ma Y, George J, Dietz-Band J, James W, Slamon DJ, Batsakis JG, et al. (1994) Cancer Res. **54,** 5675-82.

Reese D, Arboleda J, Twaddell T, et al: (1996) Proc. Am. Assoc. Cancer Res. 37: 51, (abstr 353).

Reile H, Birnbock H, Bernhardt G, Spruss T, & Schonenberger H. (1990) Analytical Biochem, **187,** 262-7.

Rodriguez GC, Boente MP, Berchuck A, Whitaker RS, O'Briant KC, Xu F, & Bast RC.Jr. (1993) Am. .J. Obstet. and Gynecol. **168,** 228-32.

Saffari B, Jones LA, el-Naggar A, Felix JC, George J, & Press MF. (1995) Cancer Res. **55,** 5693-8.

Semba K, Kamata N, Toyoshima K, & Yamamoto T.(1985) Proc. Natl Acad. of Sci. USA. **82,** 6497-501.

Sias PE, Kotts CE, Vetterlein D, Shepard M, & Wong WL. (1990) J. Immunological Methods, **132,** 73-80.

Slamon DJ, Clark GM, Wong SJ, Levin WJ, Ullrich A. and McGuire WL. (1987) Science (Washington, D.C.), **235,** 177-182.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. (1989) Science, **244,** 707-12.

Tannock IF. (1978) Cancer Treat Rep. **62,** 1117-1133.

Yarden Y. (1990) Proc. Natl. Acad. Sci. USA, **87** 2569-73.

Yonemura Y, Ninomiya I, Ohoyama S, Kimura H, Yamaguchi A, Fushida S, Kosaka T, Miwa K, Miyazaki I, Endou Y, et al. (1991) Cancer, **67** 2914-8.

Zuckier G, Tritton TR. (1983) Experimental Cell Res. **148** 155-61.

Table 1. Inhibition of SK-BR-3 cell proliferation by TSPA, and rhuMAb HER2, alone and in combination.

Fractional inhibition (Fa) of:								
TSPA (uM)	0	8.3	16.5	33.0	66.1	132.1	264.3	528.5
rhuMAb HER2 (nM)								
0	0	0.16	0.26	0.36	0.46	0.61	0.79	0.84
0.5	0.25	0.37						
1.1	0.3		0.42					
2.1	0.28			0.52				
4.25	0.32				0.58			
8.5	0.35					0.69		
17	0.38						0.82	
34	0.38							0.86

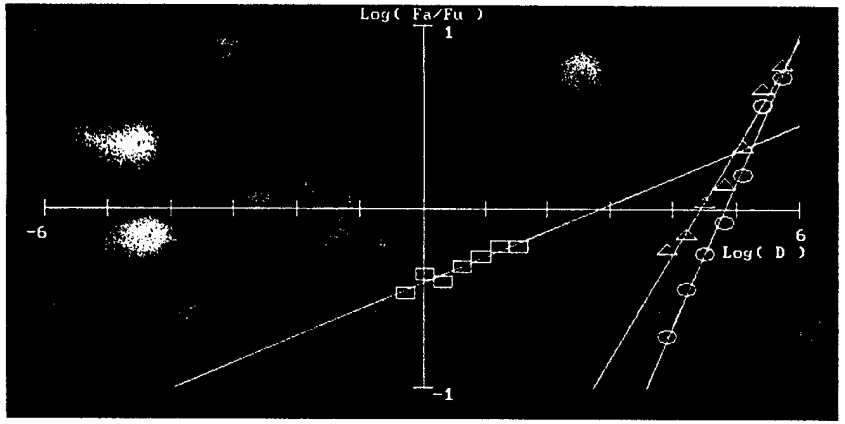
SK-BR-3 cells ($5X10^3$/well) were incubated in triplicate wells as described in the methods section.
The incubation period was 72h.

Table 2. Calculated values for the Combination Index as a function of fractional inhibition of SK-BR-3 cell proliferation by a mixture of TSPA and rhuMAb HER2.

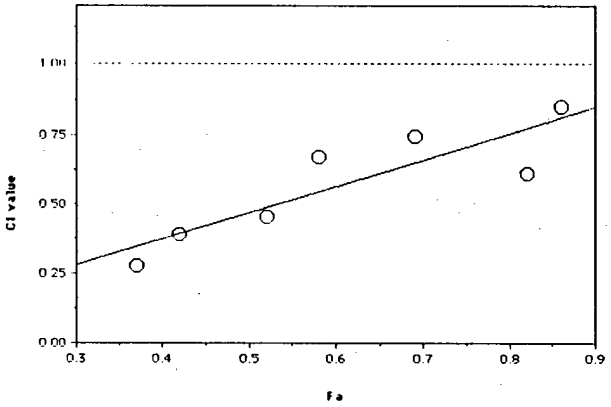
	Combination Index Values at:					Parameters:		
Drug	ED30	ED40	ED50	ED60	ED70	Dm	m	r
TSPA						66.2uM	0.81	0.99
rhuMAb HER2						675.0nM	0.15	0.96
TSPA+rhuMAb HER2	0.52	0.37	0.41	0.49	0.60	27.1uM	0.59	0.99
Diagnosis of combined effect	Synergy	Synergy	Synergy	Synergy	Synergy			

Table 3. Mean combination index values for chemotherapeutic drug/rhuMAb HER2 combinations *in vitro*.

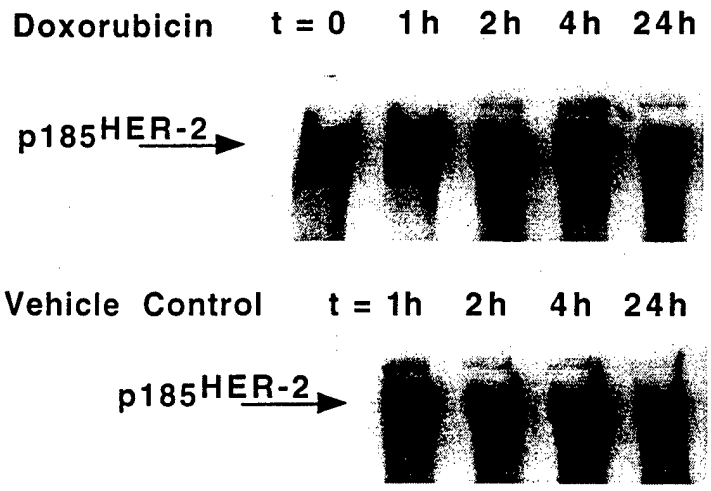
Drug	rhuMAb HER2/Drug Molar Ratio	Drug Dose Range (uM)	Combination Index (Mean ± SEM)	P Value	Interaction
TSPA	6.4×10^{-5}	$8.25 - 1.06 \times 10^{3}$	0.67 ± 0.12	0.0008	Synergy
CDDP	4.0×10^{-4}	$6.5 \times 10^{-1} - 1.7 \times 10^{2}$	0.56 ± 0.15	0.001	Synergy
VP-16	9.9×10^{-4}	$2.6 \times 10^{-1} - 6.8 \times 10^{1}$	0.54 ± 0.15	0.0003	Synergy
DOX	9.8×10^{-3}	$2.7 \times 10^{-2} - 6.9$	1.16 ± 0.18	0.13	Addition
TAX	1.4×10^{-1}	$1.8 \times 10^{-3} - 5.0 \times 10^{-1}$	0.91 ± 0.23	0.21	Addition
MTX	3.3×10^{-1}	$8.0 \times 10^{-4} - 2.0 \times 10^{-1}$	1.36 ± 0.17	0.21	Addition
VBL	1.7	$1.6 \times 10^{-4} - 3.9 \times 10^{-2}$	1.09 ± 0.19	0.26	Addition
5-FU	8.8×10^{-5}	$3.0 - 7.65 \times 10^{2}$	2.87 ± 0.51	0.0001	Antagonism

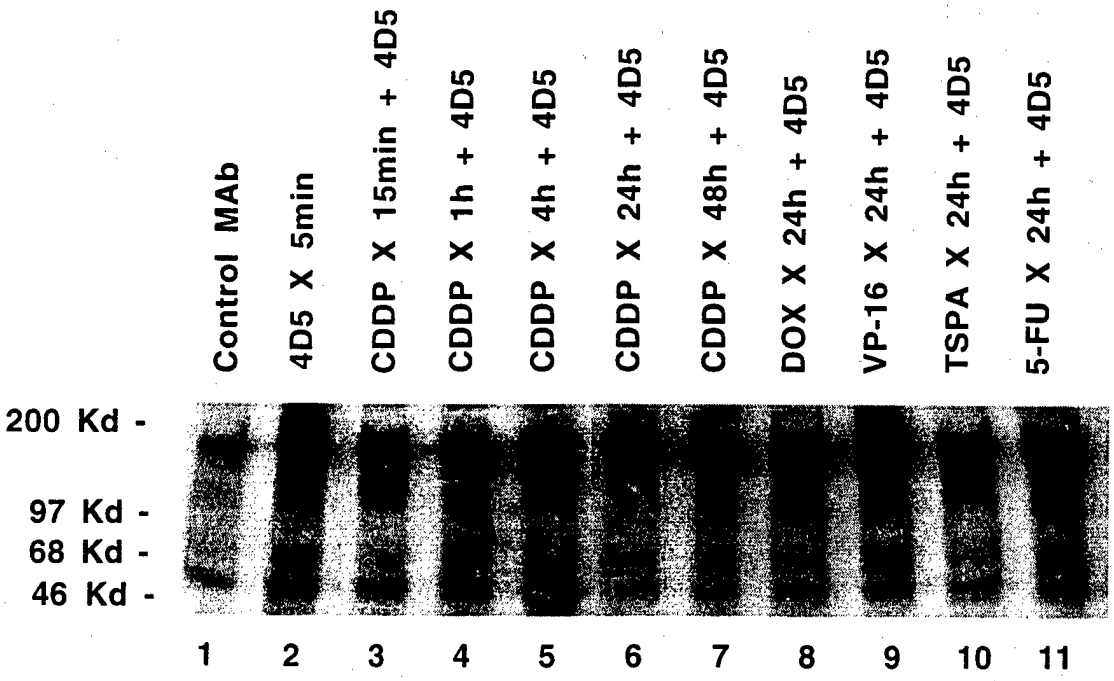

Log(Fa/Fu)
1
-6
6
Log(D)
-1

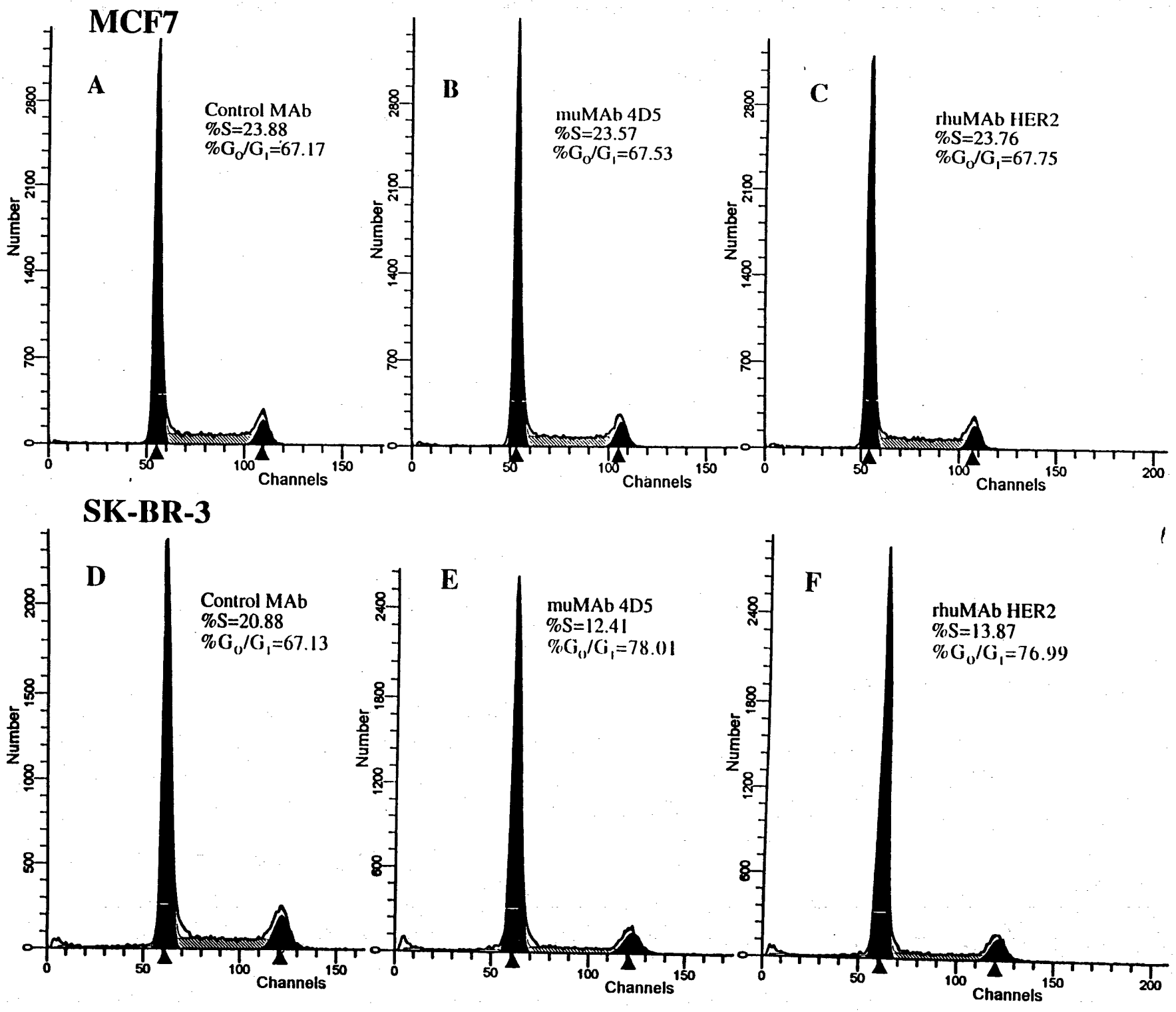
A

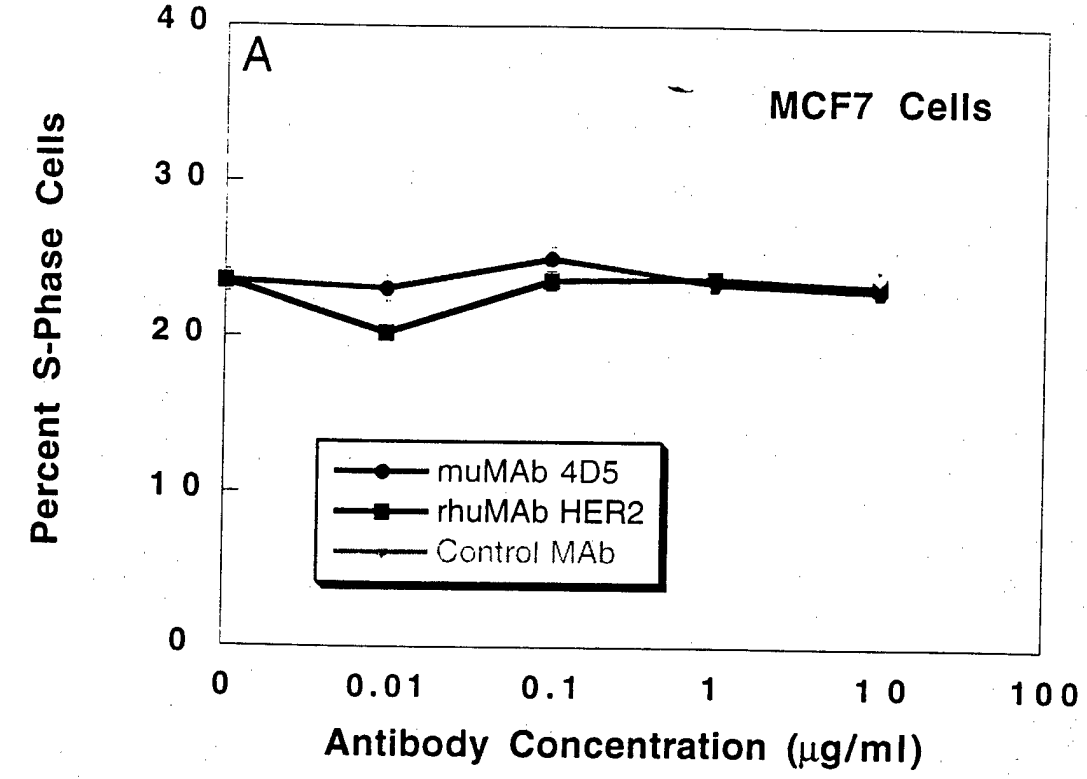

CI value
1.00
0.75
0.50
0.25
0.00
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Fa

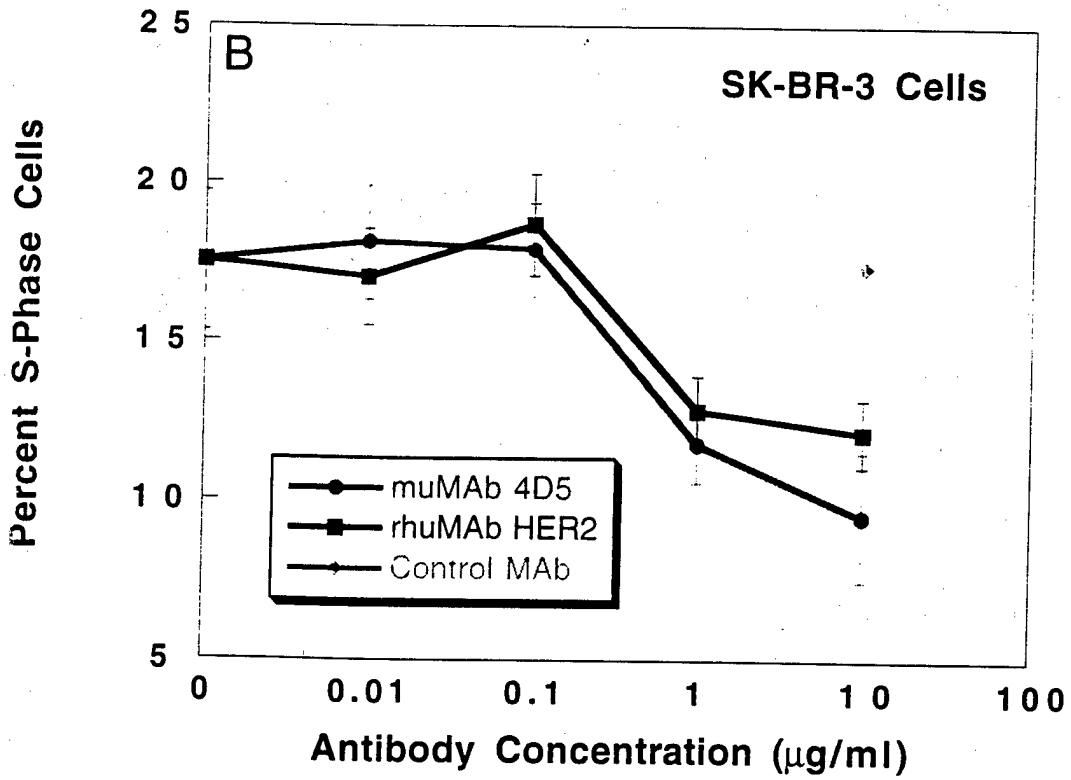
B


Doxorubicin t = 0 1h 2h 4h 24h
p185HER-2
Vehicle Control t = 1h 2h 4h 24h
p185HER-2

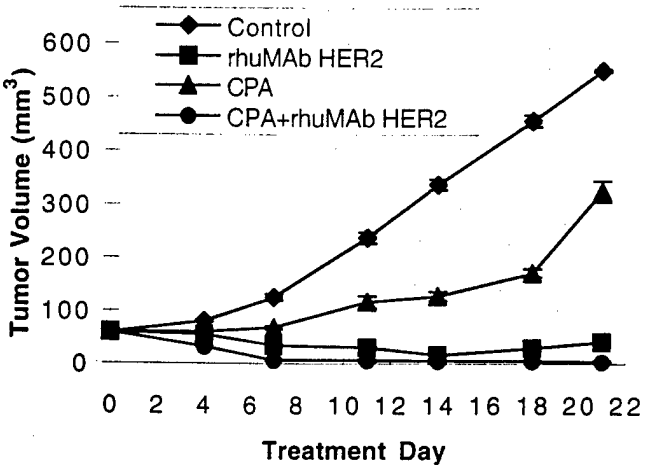

Control MAb
4D5 X 5min
CDDP X 15min + 4D5
CDDP X 1h + 4D5
CDDP X 4h + 4D5
CDDP X 24h + 4D5
CDDP X 48h + 4D5
DOX X 24h + 4D5
VP-16 X 24h + 4D5
TSPA X 24h + 4D5
5-FU X 24h + 4D5
200 Kd -
97 Kd -
68 Kd -
46 Kd -
1 2 3 4 5 6 7 8 9 10 11


MCF7
A
Control MAb
%S=23.88
%G0/G1=67.17
B
muMAb 4D5
%S=23.57
%G0/G1=67.53
C
rhuMAb HER2
%S=23.76
%G0/G1=67.75
SK-BR-3
D
Control MAb
%S=20.88
%G0/G1=67.13
E
muMAb 4D5
%S=12.41
%G0/G1=78.01
F
rhuMAb HER2
%S=13.87
%G0/G1=76.99
Number
Channels

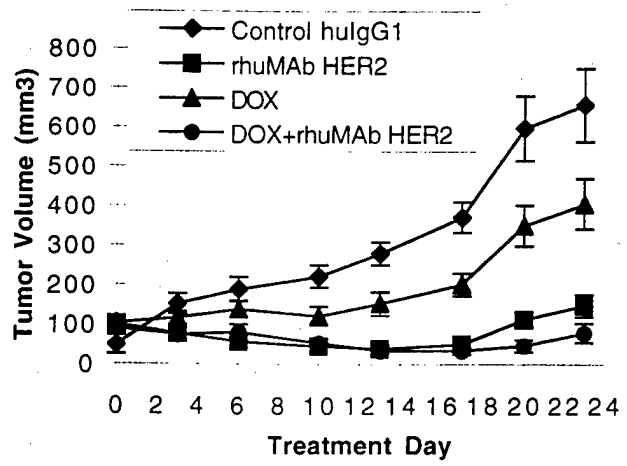

A
MCF7 Cells
Percent S-Phase Cells
40
30
20
10
0
muMAb 4D5
rhuMAb HER2
Control MAb
0
0.01
0.1
1
10
100
Antibody Concentration (µg/ml)


B
SK-BR-3 Cells
Percent S-Phase Cells
25
20
15
10
5
muMAb 4D5
rhuMAb HER2
Control MAb
0
0.01
0.1
1
10
100
Antibody Concentration (µg/ml)

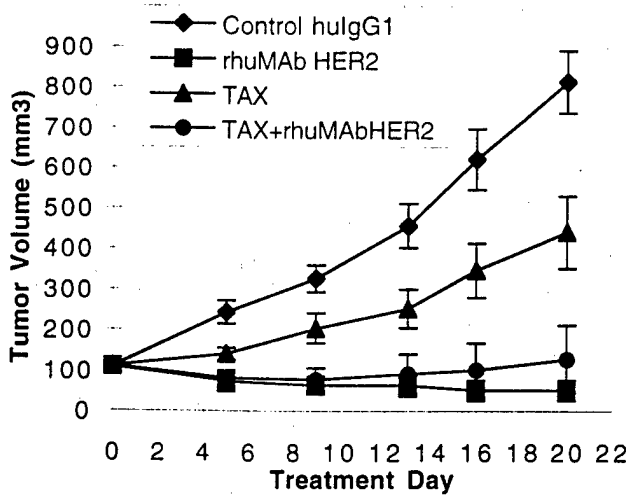
A

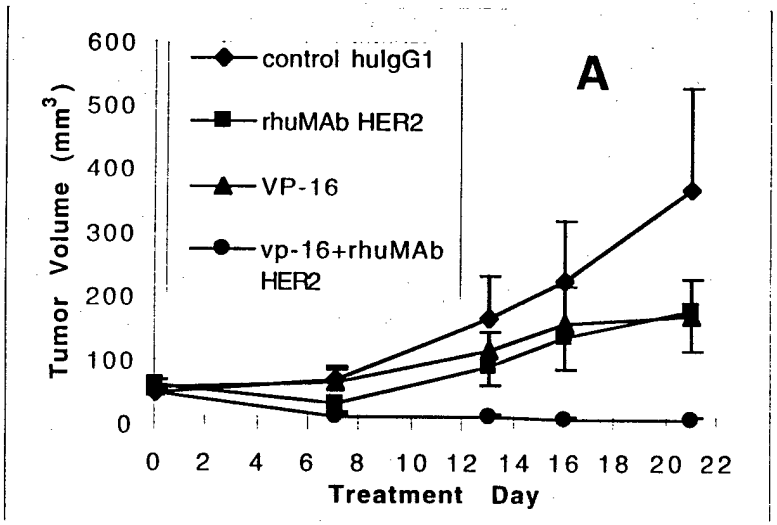

Control
rhuMAb HER2
CPA
CPA+rhuMAb HER2
Tumor Volume (mm3)
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22
Treatment Day

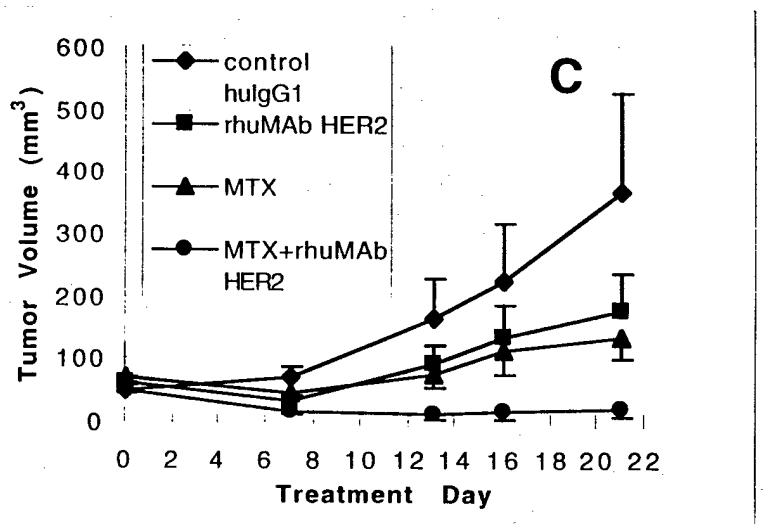
B

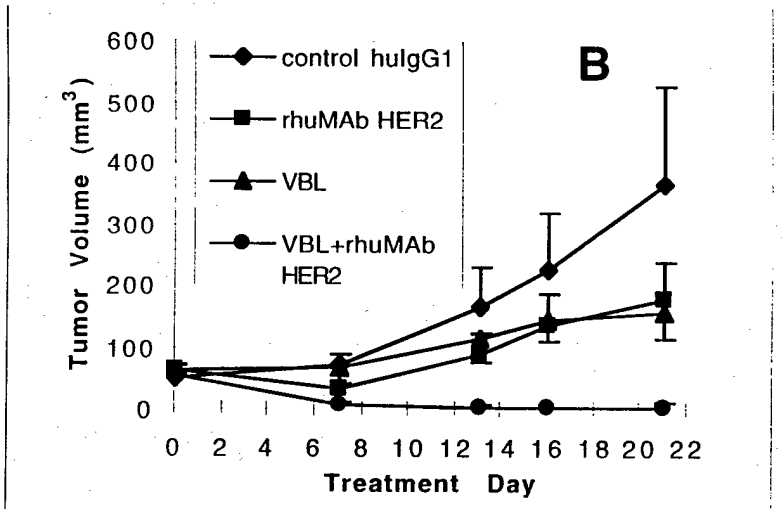

Control huIgG1
rhuMAb HER2
DOX
DOX+rhuMAb HER2
Tumor Volume (mm3)
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Treatment Day

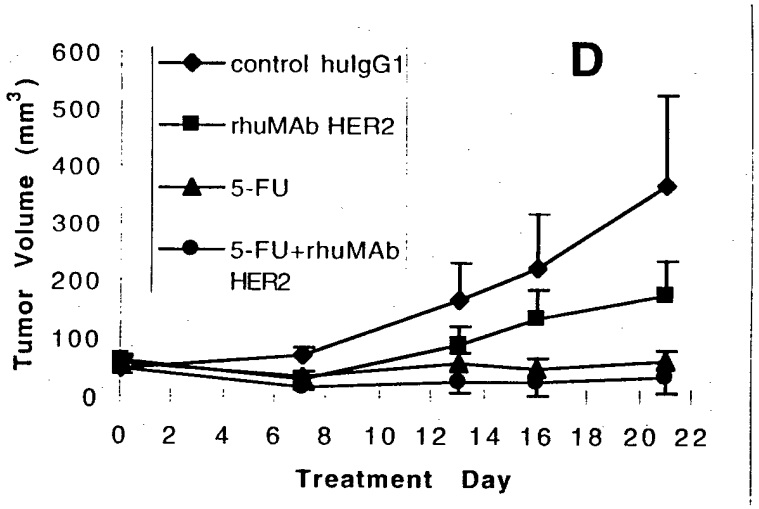
C


Control huIgG1
rhuMAb HER2
TAX
TAX+rhuMAbHER2
Tumor Volume (mm3)
900
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22
Treatment Day


A
control huIgG1
rhuMAb HER2
VP-16
vp-16+rhuMAb HER2
Tumor Volume (mm3)
Treatment Day
0
100
200
300
400
500
600
0 2 4 6 8 10 12 14 16 18 20 22


C
control huIgG1
rhuMAb HER2
MTX
MTX+rhuMAb HER2
Tumor Volume (mm3)
Treatment Day
0
100
200
300
400
500
600
0 2 4 6 8 10 12 14 16 18 20 22


B
control huIgG1
rhuMAb HER2
VBL
VBL+rhuMAb HER2
Tumor Volume (mm3)
Treatment Day
0
100
200
300
400
500
600
0 2 4 6 8 10 12 14 16 18 20 22


D
control huIgG1
rhuMAb HER2
5-FU
5-FU+rhuMAb HER2
Tumor Volume (mm3)
Treatment Day
0
100
200
300
400
500
600
0 2 4 6 8 10 12 14 16 18 20 22

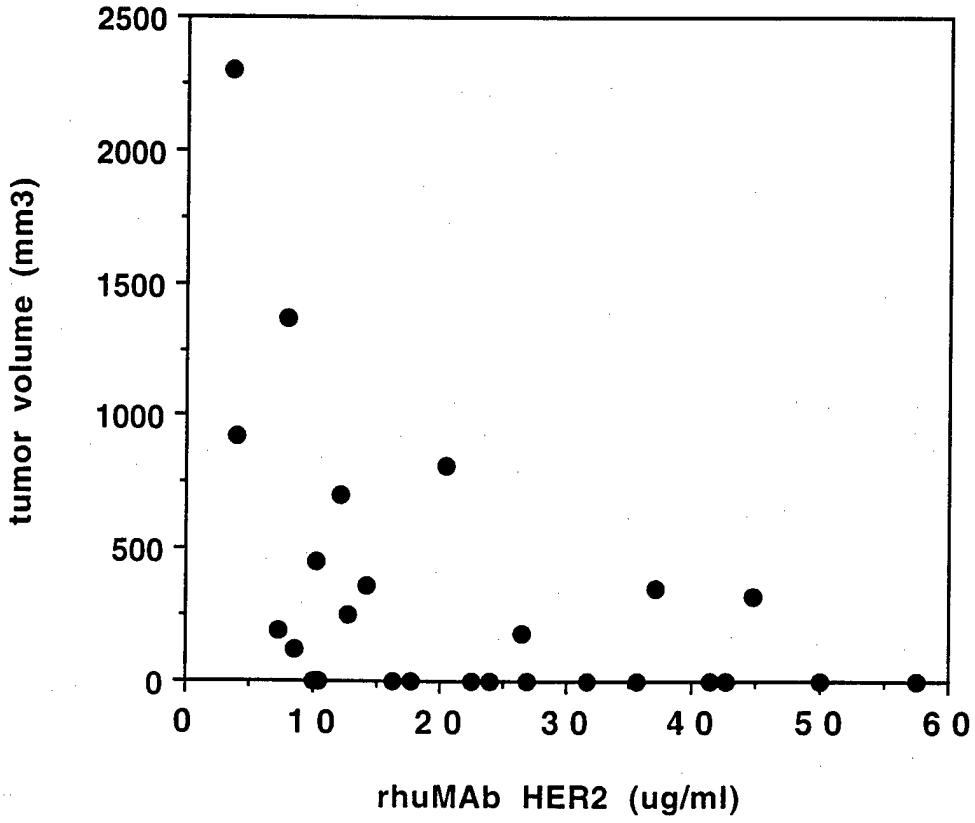

2500
2000
1500
1000
500
0
tumor volume (mm3)
0
10
20
30
40
50
60
rhuMAb HER2 (ug/ml)

Figure Legends

1 (A) Multiple drug effect plot of TSPA, rhuMAb HER2 and the combination. Fa = the fraction of SK-BR-3 cells affected by the drugs, Fu = the fraction of cells unaffected. D = drug dose. (B) Combination Index values for TSPA in combination with rhuMAb HER2 at multiple effect levels. CI values < 1 indicate synergy.

2. Expression of $p185^{HER\text{-}2/neu}$ in SK-BR-3 cells following exposure to DOX at the IC_{30} (30nM) concentration.

3. MAb 4D5-induced tyrosine phosphorylation of $p185^{HER\text{-}2/neu}$ in SK-BR-3 cells following exposure to chemotherapeutic agents at the IC_{30} concentration.

4. DNA fluorescence flow cytometry histograms of propidium iodide-stained nuclei obtained from MCF7 (A - C) and SK-BR-3 (D - F) breast carcinoma cells following treatment with control antibody 6E10, murine anti-$p185^{HER\text{-}2/neu}$ antibody 4D5, or humanized anti-$p185^{HER\text{-}2/neu}$ antibody (rhuMAb HER2) at a dose of 1 ug/ml for 72h.

5. Effect of anti-$p185^{HER\text{-}2/neu}$ MAb dose on cell cycle distribution of MCF7 (A) and SK-BR-3 (B) cells.

6. Combination treatment of MCF7/HER-2 breast carcinoma xenografts in athymic mice with rhuMAb HER2 plus CPA (A), DOX (B), and TAX (C).

7. Treatment of MCF7/HER2 xenografts with rhuMAb HER2 in combination with VP-16 (A), VBL (B), MTX (C), and 5-FU (D).

8. Relationship between MCF7/HER-2 xenograft volume and trough rhuMAb HER2 concentration in murine serum.

Contemporary Endocrinology (in press)

Oncogene Activation and Breast Cancer Progression

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1. Gene Alterations in Human Breast Cancer

Gene alterations play an important role in the origin and pathogenesis of human breast cancer. Three broad categories of gene changes that appear to contribute to tumor progression include tumor suppressor genes, repair-mutator genes and oncogenes (1). Inherited defects in some somatic genes appear responsible for development of hereditary and familial breast cancer, estimated at 1% and 5%, respectively, of all breast cancer cases. Identified alterations include mutations in tumor suppressor genes such as p53 in Li-Fraumeni syndrome (2,3). Mutations in the BRCA-1 gene at chromosome 17q21 have been documented in familial breast cancer (4). A separate locus on chromosome 13q13, BRCA-2 gene, was also associated with familial breast cancer (5,6). Recent studies suggest that BRCA-1 may represent a repair-mutator gene, a gene responsible for maintaining the fidelity of DNA duplication (7). The failure of gene surveillance can result in further alterations in gene function and, thereby, increase the mutation rate of other genes. Presumably, tumor suppressor genes and oncogenes would be prominent targets of faulty repair-mutator genes (1,7).

Oncogenes are genes directly responsible for cancer progression and often present as altered versions of proto-oncogenes that are normally involved in the control of cell growth and differentiation (1,3). In the breast cancer cell, qualitative or quantitative differences are found between the proto-oncogene and it's corresponding oncogene. The proto-oncogene can become an oncogene when a mutation in the coding region constituitively activates the biologic activity of the protein product without affecting the total amount of the product. Alternatively, a proto-oncogene can become an oncogene when excess product occurs from amplification (multiple copies) of the gene or from mutation, rearrangement, insertion or deletion of the regulatory region of gene (8). The oncogenes are, in turn, involved in the regulation of a complex series of cyclin-dependent kinases and other cell cycle modulators that determine progression through the cell division cycle (3). Other categories of genes and their products that affect tumor progression include hormonal influences and angiogenic factors, topics that are detailed in independent chapters of this volume.

2. Molecular Genetics of Breast Cancer Progression

Breast cancer progression is hypothesized to occur by an accumulated series of genetic and phenotypic changes in pathways regulating cell growth (Figure 1). Intraductal carcinoma or ductal carcinoma in situ is the earliest histologic pattern considered a breast cancer. Cells within these malignant ducts have the cytologic features of more advanced malignancy but grow within the confines of an intact basement membrane without microscopic evidence of invasion (Figure 1). Ductal carcinoma in situ appears capable of progression to invasive cancer. Inherited or somatic genetic changes in oncogenes, tumor suppressor genes, DNA repair machinery and cell cycle checkpoints lead to in situ carcinoma and subsequently to invasion and metastasis. Familial disease may bypass one or more steps in this postulated cascade. With classic cytogenetic methods and studies of loss of heterozygosity, gene regions identified as commonly rearranged, amplified or otherwise altered have been commonly detected at chromosome 1,3,6,7,8,9,11,13, 15,16, 17, 18 and 20 (3,6). Application of comparative genomic hybridization has also implicated chromosome 10,12 and 22 in the malignant process. As in most human cancers, the most common genetic abnormality in breast cancer is loss of specific chromosome arms. Loss of heterozygosity analysis of polymorphic DNA markers point to chromosomes and subregions of chromosome arms likely to harbor tumor suppressor genes. Loss of heterozygosity generally allows expression of a recessive mutant in an allele of a tumor suppressor gene by removal of a dominant normal allele, as in the case of p53 expression (1-3).

The second most common type of cytogenetic alteration in breast cancer appears to be gene amplification (3,8). Karyotype analysis and chromosome in situ hybridization approaches such as comparative genomic hybridization or micro-fluorescent in situ hybridization (FISH) point to amplified chromosomal loci likely to harbor oncogenes. The initial step in gene amplification may involve the formation of extra-chromosomal, self-replicating units termed double-minute chromosomes. These elements then become permanently incorporated into chromosomal regions and are termed homogeneous staining regions (Figure 2). An amplified genetic unit (amplicon) is

initia ch larger than the actual size of principal gene of biologic importance. Irrelevant or silen may also be coamplified with one or more expressed genes on an amplicon (3,8).

3. O ne Amplification in Breast Cancer Progression

best-established examples of amplified and functional genes for breast tumorigenesis (dom ncogenes) include the growth factor receptor, c-neu / HER-2 (c-erb B2) and the nucl scription factor, c-myc (Table 1). However, the genetic diversity of breast cancer is refle the various oncogenes implicated in breast cancer progression (3). Gene amplification occu e following loci: HER-2/neu (chromosome 17q12, 20-30% of tumors) and c-myc (8p2- of tumors). Genes encoding cell cycle kinase regulatory proteins, such as cyclin D1 [PRA D1 (chromosome 11q13)], are also commonly amplified in about 15% of human breas rs and are considered oncogenes (Table 2). Other candidate oncogenes showing ampl n at specific loci include the fibroblast growth factor receptors, FLG at chromosome 8p12 5% of tumors) and BEK at chromosome 10q26 (10-15% of tumors), the insulin-like growt or receptor, IGFR at chromosome 15q24-25 (2% of tumors), and unidentified genes at chron 13q31, 17q22-24 and 20q11-13.2 (see Table 2; 3). AIB1, a steroid receptor coact mplified in approximately 10% of human breast cancers, was recently identified at chron 20q12. Altered expression of this protein may contribute to development of steroid-deper ancers (9).

the exception of c-myc and PRAD1/CYCLIN D1, gene amplification in breast cancer comm nvolves one of several growth factor receptors. Growth factor receptor pathways play a criti e in human breast cancer progression (10). In particular, members of the epidermal growtl r receptor (EGFR) family of growth factor receptors (EGFR, HER-2/ neu / erb B2, HER-3 B3, HER-4 /erb B4) appear to play a critical role in breast cancer progression. Recept r HER-2, HER-4 and EGFR have up to 80% sequence homology, predominantly in the tyr kinase domain, and encode transmembrane glycoproteins with tyrosine kinase activity that ap essential for the signaling function of these molecules (Figure 3). These receptors

transmit growth and differentiation signals to the intracellular machinery (*ras* / MAP kinase cascades) in response to specific ligands such as heregulins or EGF. In contrast, HER-3 receptor has substitutions in several important amino acids in its tyrosine kinase domain and may have reduced or absent enzymatic activity (10). However, HER-2/neu receptor can form functional receptor heterodimers with HER-3 and with each of the other EGFR family members (10,11).

Although several members of the EGFR family appear to be overexpressed in breast cancer (6,10,11), amplification and overexpression of HER-2 gene has been studied most extensively. The gene known as neu, erb B2 or HER-2 was first identified as a dominant transforming gene activated in chemically-induced rat neuroectodermal tumors (12). Although the original oncogenic alleles of neu were found to have a single point mutation in the transmembrane domain of the product (13), the receptor is activated in human breast cancer only through amplification and overexpression of the wild-type gene. Reports by Slamon and colleagues (14,15) presented ideal studies of the changes in HER-2/neu oncogene expression in breast cancer specimens. The investigators used Southern, Northern and Western blots and immunohistochemistry for HER-2/neu detection in 187 breast tumor specimens in order to analyze the amount of HER-2/neu expression at gene, RNA transcript and protein levels. The latter work and ensuing studies established that 20-30% of breast tumors have amplification of HER-2/neu gene and overexpress the encoded protein, a 185 kDa transmembrane tyrosine kinase receptor for growth factors (3,10, 11,14,15). The temporal occurrance of HER-2 gene amplification in breast tumor progression has also been studied in several recent investigations. In one notable work, amplification of HER-2/neu was assessed by fluorescent in situ hybridization (FISH) in a range of proliferative and malignant ductal lesions of the breast, allowing for interphase analysis of gene copy number on a cell by cell basis (Figure 4; 16). Using the latter approach, HER-2/neu amplification was found to be restricted to ductal carcinoma in situ, predominantly in comedo-type, and to invasive carcinoma and was not detected in non-malignant intraductal proliferations such as ductal hyperplasia and atypical ductal hyperplasia. Amplification was almost exclusively restricted to in situ and invasive carcinomas of high histologic grade (16). The work suggests that HER-2/neu gene amplification is

an early event in the development of high grade ductal malignancies, but oncogene alterations are not evident in early hyperproliferative or premalignant atypical ductal lesions (16-18). Independent work shows that expression of HER-2/neu is maintained during progression from intraductal to invasive phases of growth in the same tumor tissue (19-21). Overexpression of HER-2/neu is also maintained in metastatic lesions, suggesting a continuing function for HER-2/neu (19). However, HER-2/neu overexpression likely represents only one histomorphologic pathway of breast tumorigenesis. A significant subset of breast carcinomas likely do not develop from HER-2-overexpression, and independent and/or complementary molecular events are required to explain these alternate pathways to malignancy (Table 1 and 2). As noted above, the development of cancer is a process that involves not only the activation of oncogenes but also the dysregulation of tumor suppressor and repair-mutator gene function (1-3, 17; Figure 5).

4. Clinical Implications of HER-2/neu Gene Overexpression in Breast Cancer

HER-2/neu overexpression is associated with poor prognosis in patients with node-positive and node-negative breast cancers (3,8,14,15,21). In addition, overexpression of HER-2 receptor is associated with a poor clinical response to certain chemotherapeutic (22-24) and antihormonal drugs (25-29). There is currently ongoing debate about the efficacy of standard chemotherapy in breast cancer patients whose tumors have high levels of HER-2 receptor (3,30), but several studies suggest that patients whose tumors overexpress HER-2 respond worse to antihormone treatment (31). Further well-designed clinical trials should help to clarify these important problems.

Since activation of the HER-2/neu signal transduction pathway correlates with the ability of HER-2/neu to transform breast epithelial cells, the occurrance of HER-2/neu gene in human breast cancers has significant therapeutic implications (8,10) . Monoclonal antibodies directed to the extracellular domain of HER-2/neu receptor reduce the proliferation of breast cancer cells that overexpress HER-2 receptors, thus providing a rationale for the therapeutic targeting of this growth pathway. In addition, monoclonal antibodies to HER-2/neu receptor have been found to sensitize breast cancer cells to chemotherapeutic agents that elicit damage to cellular DNA (32,33).

A recombinant humanized monoclonal antibody to HER-2 receptor is currently in Phase III clinical trials alone and in combination with chemotherapeutic drugs (34). Depending on the latter results, treatment with HER-2 specific monoclonal antibodies alone or in combination with other agents may prove to be an important new therapy for breast cancer.

5. The Role of the Epidermal Growth Factor Receptor in Breast Cancer

The epidermal growth factor receptor is a 170-kd transmembrane receptor with tyrosine kinase activity (35). EGFR shares considerable sequence homology with other members of the type I receptor tyrosine kinase family, HER-2/neu, HER-3, and HER-4 (Figure 5; 35-37). The structural motifs in this family include four conserved domains: two cysteine-rich extracellular domains which are critical for ligand binding, a hydrophobic transmembrane domain, and a cytoplasmic kinase domain. In addition to the kinase activity of the cytoplasmic portion of EGFR, the phosphorylated form of EGFR has high-affinity recognition sites for Grb-2 (growth factor receptor bound-2), SHC (38,39), and SH2 (src homology type-2) domain-containing proteins (such as those found in phospholipase C-γ). As an adaptor protein forming a complex between activated tyrosine kinases and *ras*, Grb-2 serves a crucial link between EGFR and SOS, a *ras* GTP/GDP exchange protein (40-41). Formation of the EGFR /Grb-2/ SOS complex serves to catalyze the *ras* -activated exchange of GTP for GDP. In a simplified model of receptor tyrosine kinase signal transduction (Figure 5), activated *ras* stimulates *raf* kinase which, in turn, phosphorylates MEK (a MAP-kinase kinase) and MAP kinases leading to regulation of the function of nuclear transcription factors that direct mitogenesis or differentiation. The complexity of EGFR signaling is amplified by the several ligands which bind to EGFR, including EGF, TGF-α, amphiregulin, and cripto-1, and by the capability of EGFR to transactivate other type-I tyrosine kinases including HER-2/*neu* and HER-3 receptors (Figure 5; 42).

The gene encoding EGFR, c-erb-B, is localized to chromosome 7 and is homologous to the v-erb-B oncogene. Although transfection of EGFR alone is insufficient for transformation of mammalian cells, co-transfection of EGFR with one of it's activating ligands, such as EGF or

TGF-α, does result in transformation, thus establishing EGFR as a proto-oncogene. A role for EGFR in the pathogenesis of breast cancer is suggested by the fact that the receptor and some of it's ligands are overexpressed in a significant fraction of breast cancers compared to expression levels seen in normal breast tissues (43,44). In contrast to the c-erb B-2 gene, the c-erb B proto-oncogene is generally not amplified in breast cancers, with overexpression due to an increase in production of the protein product. The clinical importance of EGFR overexpression was first suggested by Sainsbury et al. (45) who reported that EGFR is an independent predictor of early relapse and death in patients with breast cancer. However, several following reports have offered widely conflicting results on the prognostic significance of EGFR expression in breast cancer. Comprehensive reviews of the latter cohorts (46,47) conclude that: 1) based on results from 40 studies comprising 5,232 patients, EGFR overexpression is found in about 45% of all breast cancers (range 14-91%); 2) EGFR overexpression is often associated with shortened relapse-free, but not overall, survival by univariate analysis; 3) the prognostic significance of EGFR overexpression is lost on application of multivariate analyses that control for other prognostic variables such as c-erb B-2 overexpression; 4) there is a lack of statistical association between EGFR and tumor size, lymph node status, tumor cell differentiation / grade, or menopausal status; 5) overexpression of both EGFR and HER-2/neu portends a particularly poor prognosis; and 6) there is a highly significant inverse correlation between EGFR expression and steroid receptor (ER, PgR) expression (47). Further, there appears to be an inverse association between EGFR overexpression and the response to antiestrogen therapy in breast cancer (48). These combined data suggest that, while EGFR expression may not be a useful prognostic factor in breast cancer, it may be a useful predictive factor for response to hormonal therapy. Support for this hypothesis is provided by laboratory experiments showing that transfection of EGFR into hormone-dependent breast cells results in hormone independence, loss of ER and PgR expression and acquired resistance to tamoxifen (49,50). Indeed, emerging data also suggest a potential role for EGFR in resistance to chemotherapy. Doxorubicin-resistant MCF-7 cells have increased expression of EGFR, and transfection of EGFR into breast cells confers resistance to certain chemotherapeutic

drugs (51). A main difficulty in the use of EGFR as a predictive marker in breast cancer is the lack of standardization in the measures of EGFR protein in tumor samples. Popular methods include immunohistochemical and radioligand binding assays. Unfortunately, antibodies, labeling approaches, and cut-off values to discriminate positive from negative results differ significantly in each study. Until uniform standards are established, the precise role of EGFR expression in breast cancer progression will remain uncertain. It is likely that tumor expression of EGFR ligands and other type-I receptors transactivated by EGFR contribute to the clinical significance of EGFR expression and should be evaluated in parallel in future studies. The potential role of EGFR in tumor initiation or transition from pre-invasive to invasive malignancy also remains to be assessed.

6. The Role of c-myc in Breast Cancer

The cellular homologue of v-*myc*, c-*myc*, is a 439-amino acid nuclear phosphoprotein that functions as a transcription factor. It is often overexpressed in breast cancer with amplification of the c-*myc* gene on chromosome 8q24 (52). The structure of c-*myc* includes an amino-terminal transcription activation domain, a basic DNA-binding domain, a helix-loop-helix motif, and a leucine-zipper motif (Figure 5). The later two motifs are responsible for the formation of both homo- and hetero-dimers. Heterodimeric complexes between *myc* and an 18-kd helix-loop-helix protein, *max* , bind specifically to E-box DNA sequences (CACGTG), resulting in transcription activation. In contrast, *max* homodimers inhibit transactivation, and *max* complexed with another helix-loop-helix protein, *mad*, also inhibits transcription in conjunction with the corepressor protein, Sin3. *Myc* expression is induced by a variety of growth factors, including EGF, TGF-α, IGF-I, heregulin, and by steroid hormones, such as estradiol and progesterone (53-56). A specific estrogen-responsive region of the c-*myc* gene has been found (57), and constitutive up-regulation of c-*myc* expression is noted in ER-negative breast cells (58). Inhibition of estrogen-induced expression of c-*myc* protein by an antisense oligonucleotide results in arrest of estrogen-stimulated cell proliferation (59). C-*myc* expression is attenuated by antiestrogens in ER-expressing breast cell lines (57), and also by TGF- β or oncostatin M, factors that inhibit the

growth of mammary epithelial cells *in vitro* (60,61). This regulation of c-*myc* expression by estrogen and by mitogenic growth factors that are known to be expressed in breast tissues suggests a role for dysregulation of c-*myc* in the malignant transformation of breast epithelia.

The transformation of normal epithelial cells by c-*myc* requires cooperation with other oncogenes or peptide growth factors. For example, human mammary A1N4 cells transfected with c-*myc* could only form colonies under anchorage-independent conditions with the addition of exogenous EGF, TGF-α, or bFGF (62). This suggests that c-*myc* overexpression alone is not sufficient for tumorigenesis. In support of this hypothesis, transgenic mice produced by microinjection with an MMTV-LTR-c-*myc* construct into pronuclei of fertilized eggs results in mice which develop mammary tumors but only after a long latency period and/or multiple pregnancies. Such latency periods suggest that other genetic alterations must take place in addition to c-*myc* overexpression in order to result in malignant degeneration of breast epithelia (63-65). The fact that double transgenic strains in which c-*myc* is co-overexpressed with v-Ha-*ras*, c-*neu*, or TGF-α results in a shorter latency to onset of breast tumors also supports this view (66-68).

A summary of 30 studies published between 1986 and 1996 on the incidence and prognostic significance of c-*myc* gene amplification in breast cancer was recently reported by Watson et al. (69). In this analysis, encompassing over 5,000 breast tumors, the amplification rate was about 15% (range 1% to 33%). Wide variability in the results is likely due to technical differences in patient selection, cut-off points for gene amplification, contamination of tumor cell populations by stromal cells (in studies using Southern blot techniques), and the use of different control genes. Nonetheless, the incidence of c-*myc* amplification in these studies is near the approximate 20% incidence of c-*myc* amplification found in breast carcinoma cell lines (70). Genetic rearrangements of c-*myc* are found infrequently in breast cancer (71). Despite considerable variability in correlations between c-*myc* amplification and other established prognostic factors in breast cancer, there is a relatively consistent association detected with pathologic grade (69). In addition, other reports have demonstrated an association between c-*myc* amplification and shortened relapse-free or overall survival, lymph node status, DNA ploidy,

steroid hormone receptor status, cathepsin D expression, and inflammatory breast cancer (52). With the exception of one study (72), co-amplification of c-*myc* and c-erb B-2 appears to be a very infrequent occurrance. In one provocative report, a significant association between c-*myc* amplification and LOH on chromosome 1p was noted, suggesting the possibility of a tumor suppressor gene at this locus that, when lost, may facilitate c-*myc* amplification (73). Overexpression of c-*myc* in the absence of gene amplification also occurs in breast cancer and, although there is general agreement in these studies that c-*myc* expression is increased in breast tumor cells relative to adjacent normal cells, it remains unclear what impact this event may have on prognosis. There is recent evidence that N-*myc* protein is also overexpressed in breast carcinomas in the absence of gene amplification, and this finding may correlate with tumor stage, grade, and clinical outcome (74).

7. The *Ras* Signal Transduction Pathway in Breast Cancer

The three human *ras* proto-oncogenes encode four homologous 21-kd proteins: H-*ras*, K-*ras* 4A, K-*ras* 4B, and N-*ras* (75-78). As shown in Figure 5, *ras* plays a key role as an intermediate for signal transduction initiated by ligand binding of receptor tyrosine kinases. Activated *ras* targets mitogen-activated serine/threonine protein kinases (MAP kinases) via *raf* and MEK. MAP kinases, in turn, translocate to the nucleus where they regulate the activity of nuclear transcription factors. *Ras* localization to the cell membrane is critical for it's function, and *ras* undergoes a series of post-translational modifications which result in a mature form of the protein which is membrane-associated. *Ras* is initially prenylated, undergoes proteolytic cleavage of three C-terminal amino acid residues, and then undergoes methylation of the C-terminal carboxyl group of the prenylated cysteine residue exposed by proteolysis. Finally, *ras* proteins may be further modified by palmitoylation to stabilize membrane association (75). The activity of mature *ras* proteins is regulated by binding of guanine nucleotides, such that GTP-bound *ras* is activated and GDP-bound *ras* is inactive (76). Coordination of the phosphorylation of *ras* -bound guanine nucleotides is accomplished by guanine nucleotide exchange factors, such as SOS, and GTP-ase

activating proteins (GAPs). Oncogenic activation of *ras* by point mutations in critical regions that govern *ras* -GDP/GTP cycling render *ras* -GTP resistant to GAP. *Ras* is constitutively activated by such mutations. Although carcinogen-induced mammary cancers in rats frequently exhibit *ras* mutations, point mutations of *ras* are found in less than 5% of sporadic human breast carcinomas (77). This low incidence of *ras* mutations does not exclude the possibility that alterations in the activity of normal *ras* proteins might be intimately involved in the pathogenesis of breast cancer. Indeed, several lines of evidence point to a role for *ras* activation in the emergence of breast malignancy. First, overexpression of normal H-*ras* protein has been reported in human breast tumors (78,79). Transfection of activated *ras* into MCF-7 breast carcinoma cells also increases tumorigenicity (80) and oncogenic *ras* transfection into normal breast epithelial cells (MCF-10A) results in cellular transformation (81). Transgenic mice with mutant *ras* expression directed to breast tissue develop mammary tumors, and there is cooperation with other oncogenes such as c-*myc* in double transgeneic mice which develop mammary tumors at an even faster rate (68). H-*ras* rare alleles, consisting of a variable number of tandem repeats of a 28 base-pair region capable of binding NF-KB transcription regulatory proteins, may also be associated with an increased risk of breast cancer (82). Recently, data from Migliaccio et al. (83) showed that estradiol can activate the p21*ras* -MAP kinase pathway in MCF-7 breast cells, possibly via activation of c-*src* protein (83). Such data implicate *ras* as a possible intermediate for estrogen in breast cells. Our understanding of the role of *ras* proteins in initiation or progression of breast cancer is hampered by the fact that there is no reliable method available for measurement of *ras* activity in premalignant or malignant breast tissues. Several investigators have examined *ras* expression levels in malignant breast tissues and find that increased expression levels are detectable in 55-71% of cases. However, expression of *ras* does not appear to correlate with other clinicopathologic variables or with patient outcome (84). It is possible that studies of new chemical agents that target and disrupt *ras* will help to further elucidate the role of *ras* signal transduction in breast neoplasia (85).

8. Amplification of Chromosome 11q13 and Evidence for Cyclin D1 and Int-2/FGF-3 Amplification in Breast Cancer

The earliest studies of the chromosome 11q13 region in breast cancer were driven by the observation that it's mouse homologue is a frequent site for integration by the mouse mammary tumor virus (MMTV). The observation that this region is sometimes amplified in breast cancers prompted a search for proto-oncogenes in this region. The MMTV integration site, designated int-2, involved a segment of DNA harboring two closely-linked polypeptide growth factors, FGF-3 and FGF-4, which have oncogenic potential and may afford a selective growth advantage for cells with 11q13 amplification. However, neither of these genes are expressed in normal mammary epithelia, and, in human tumors with 11q13 amplification, there is no concordant increase in FGF-3 or FGF-4 transcripts. Thus, it appears the latter genes may be silent passengers in this specific amplicon rather than genes with significant amplification /overexpression leading to an aberrent increase in protein activity as seen with other amplified oncogenes such as c-erbB-2. These findings prompted further inspection of the 11q13 amplicon to identify new candidate oncogenes.

Other lines of evidence pointed to this region as harboring an oncogene because the segment is the target of the t (11;14)(q13,q32) translocation in mantle-cell lymphoma, and, in parathyroid adenomas, an inversion of part of chromosome 11 fuses the 11q13 region to the parathyroid hormone gene on 11p15 (86,87). Ultimately, cyclin D1 was recognized as the leading candidate gene operative on the 11q13 amplicon (88). Cyclin D1 was isolated by differential screening of cDNAs from 11q13 amplified versus non-amplified libraries. Cyclin D1 expression was induced by various growth factors, and immunostaining localized cyclin D1 protein to the cell nucleus (88). It was recognized that the yeast homologue of cyclin D1 was able to rescue the G1-S transition in yeast cells that were deficient in G1 cyclins, and sequence analysis revealed homology of the human product to other cyclin proteins. Co-immunoprecipitation experiments demonstrated association of cyclin D1 with cyclin-dependent kinases resulting in a complex which is able to phosphorylate and inactive p105 Rb and p107 Rb-related proteins (88). Further, it is noteworthy that cyclin D1 -/- knockout mice demonstrate absence of lobuloalveolar structures in breast tissues during terminal differentiation (89). In a transgenic mouse model, under the control of the MMTV promoter, cyclin D1 overexpression in mammary tissues results in hyperplasia and

neoplasia (90). However, when driven by the immunoglobulin enhancer, mice do not develop overt lymphomas unless they are crossed with other oncogenes such as *myc* or *ras* (91), suggesting that factors other than cyclin D1 overexpression alone are required for the transition from benign to malignant growth. This hypothesis is supported by transfection studies in mammalian cells which demonstrate a lack of transformation and an overall decrease in cell viability following cyclin D1 transfection, despite a decrease in the G1- S transit time (92).

In human breast cancer, amplification of 11q13 has been well studied (reviewed in 93). It is amplified in approximately 5% - 23% of breast tumors, with most studies detecting amplification rates of 15 -20%. Amplification of this region is consistently accompanied by overexpression of cyclin D1, but emerging evidence suggests that the protein product is much more frequently overexpressed than would be predicted based on the observed amplification rate in breast cancer. Thus, other mechanisms of cyclin D1 dysregulation may be operative in this malignancy (94,95). In terms of the prognostic significance of cyclin D1 amplification, several observations are noteworthy. There is clear evidence that cyclin D1 overexpression is positively correlated with expression of the estrogen receptor (94-96). In some, but not all, studies, amplification was associated with lymph node involvement and/or adverse prognosis. In a recent comprehensive study of cyclin gene amplification and overexpression in breast cancer involving a series of 1,171 breast tumors, cyclin D1 amplification was prevalent in non-comedo type ductal carcinoma *in situ* , suggesting that this amplification event may occur relatively early in the neoplastic process (96). Higher rates of amplification in lobular as opposed to ductal breast carcinomas were also found. Further, cyclin D1 was frequently overexpressed in the absence of gene amplification, and no amplification of cyclins A, D2, D3, and E was found in human breast cancers (96). Additional studies have evaluated protein expression of cyclin D1 in breast carcinomas, and, in contrast to work showing an adverse prognosis associated with 11q13 amplification, cyclin D1 protein overexpression paradoxically identifies a patient subset with a more favorable prognosis. The latter finding may be influenced by the fact that many of these cases are ER-positive and, thus, expression of cyclin D1 may be due to induction by estrogens in malignant breast tissue (95).

Some data on the expression of cyclin E protein also suggests a possible role for this protein in tumorigenesis. Porter *et al.* (97) reported that high cyclin E levels portend a poor prognosis, even in node-negative breast cancer patients. However, in the latter analysis, the investigators did not control for ER expression. Using a more comprehensive, multivariate statistical model to control for ER expression, Nielsen *et al.* (98) found that the prognostic value of cyclin E overexpression correlated strongly with an inverse correlation between ER expression and cyclin E levels.

9. Other Candidate Oncogenes in Breast Cancer Pathobiology

9.1 *Cathepsin D*

Many enzymes capable of degrading extracellular matrix, such as matrix metalloproteinases, cathepsins, and plasminogen activators have been implicated in tumor progression and metastasis (99-101). Cathepsin D is a lysosomal acid protease whose production in breast cancer cells is stimulated by estrogen (102). Following translation, pro-cathepsin D is proteolytically cleaved to an active form (103). The ability of the active species of cathepsin D to degrade extracellular matrix and to activate other proteolytic enzymes suggests a potential role for this protein in breast cancer invasion and metastasis (99,103). Overexpression of cathepsin D in transformed cells enhances their malignant phenotype and metastatic potency. Further, cathepsin D has mitogenic activity, and it may act in an autocrine or paracrine fashion to promote tumor cell proliferation (103,104). Although initial clinical studies suggested that cathepsin D may provide significant prognostic information in patients with breast cancer, subsequent investigations have been conflicting (105). Whether or not cathepsin D expression has prognostic or predictive value in breast cancer remains highly controversial. Despite a plethora of published work, data to date are too contradictory to draw meaningful conclusions. Differences in published studies are due to an unparalleled variety of assays, reagents, approaches and arbitrary clinical cut-off values. Recent evidence from immunohistochemical studies suggest the further problem that an abundance of the cathepsin D found in tumor specimens may reside in the stromal cell compartment rather than in tumor cells. Some of the latter studies suggest that expression of cathepsin D by host fibroblasts

and macrophages has prognostic significance in breast cancer and that such expression may represent the host response to tissue damage caused by advancing malignant cells (106). Evidence from large cohorts of breast cancer patients suggest that determination of total cathepsin D in cytosol extracts from whole tumor specimens (tumor + stroma) has no prognostic utility (105,107, 108). It is likely that only use of in situ hybridization methods or in situ immunohistochemical localization with monoclonal antibodies to cathepsin D or pro-cathepsin D will help to resolve the controversy on the prognostic utility of cathepsin D. Finally, evaluation of the role of related cathepsins in breast cancer may prove worthwhile (109).

9.2 *Ornithine Decarboxylase*

Ornithine decarboxylase (ODC) is the first enzyme involved in the polyamine biosynthetic pathway. The polyamines, putrescine, spermidine and spermine, are present in all cells, and levels are tightly regulated by ornithine decarboxylase (110), S-adenosylmethionine decarboxlyase, a rate limiting enzyme in spermidine and spermine biosynthesis in some cell types (111), and by enzymatic degradation of polyamines or excretion via transport proteins (112). Treatment of estrogen-responsive MCF-7 breast cancer cells with estradiol results in induction of ornithine decarboxylase gene expression (110). This induction can be augmented by the addition of IGF-I and insulin. Polyamines may then exert growth regulatory effects by facilitating the interaction of the estrogen receptor complex with DNA and by regulating the expression of estrogen-inducible genes (113-114). Growth-stimulatory effects of estradiol can be inhibited by DL-α-difluoro-methylornithine (DFMO), an irreversible inhibitor of ODC (115). Similarly, growth-inhibitory effects of tamoxifen can be reversed by the addition of polyamines, and tamoxifen has been shown to decrease ODC expression and activity (116). Cell transformation by carcinogens, viruses, or oncogenes is often accompanied by constitutive activation of ornithine decarboxylase (117). In breast cancers specimens, malignant tumor cell populations have higher levels of polyamines than surrounding normal tissues (118). Recent studies indicate that the ornithine decarboxylase gene is a transcriptional target for c-*myc* and c-*fos* (119,120). A role for ODC in tumorigenesis is

supported by the transformation of NIH/3T3 fibroblasts on transfection with ODC cDNA (121). Additional studies link increased polyamine biosynthesis with an aggressive breast cancer phenotype (122). Despite these findings, ODC overexpression in transgenic mice was not found to induce murine tumors (123). Although enzyme activity increased in almost all tissues, polyamine pools increased only in brain and testis, underscoring the complex regulation of polyamine pools by mechanisms independent of ODC expression (123). Whether or not ODC has significant independent prognostic significance in breast cancer remains to be proven. However, targeting polyamine biosynthesis as a therapeutic strategy for breast cancer may prove useful (110).

9.3 *Detection of MMTV-like Sequences in Breast Cancer*

Studies of animal oncogenic retroviruses have been fundamental to the discovery of human cellular proto-oncogenes (3,6,11). As noted above, MMTV is an agent associated with a high incidence of breast cancer in mice. It acts as an insertional mutagen and, on insertion into chromosomal DNA, activates genes not expressed in normal mammary tissue. Although efforts to demonstrate the presence of viruses in human breast cancer have often yielded contradictory results, several lines of evidence suggest a potential association between MMTV-like virus and human breast cancer. MMTV*env* -related antigenic reactivity has been detected in breast tumor tissue sections as well as in human milk, breast tumor cells in culture and patient serum (124-126). Sequence homology to MMTV has been noted in human DNA under low stringency conditions, and RNA related to MMTV has been detected in human breast cancer cells (127,128). In addition, breast cancer patients show viral-specific T-cell responses to MMTV (129), and viral particles have been detected in human breast cancer cell lines and in monocytes from affected patients (130, 131).

Using PCR technology, Pogo and Holland identified a 660-bp sequence of the MMTV*env* gene in 39% of 335 breast cancer samples. In contrast, this PCR product was only detected in 1.6% of 121 normal breast tissue specimens from reduction mammoplasty (132). The product could not be amplified from lymphocytes from breast cancer patients or from other human cancers or cell lines. Sequencing of this PCR product revealed 95-99% homology to the MMTV*env* gene

but not to other known human endogenous retroviruses. Using RT-PCR analysis, 65% of these cases had evidence for transcription of these sequences. These investigators were also able to identify a 630-bp segment with high homology to the MMTV-LTR. This segment contained both a glucocorticoid-responsive element and MMTV superantigen domains (132). Taken together, these data suggest the possibility of an MMTV-like virus associated with a significant fraction of human breast cancers. The notion that viruses may play a role in the pathogenesis of human malignancies was recently promoted by the discovery of a member of the herpes virus family associated with progression of Kaposi's sarcoma and multiple myeloma (133). Elucidation of a possible viral etiology for breast cancer could have profound implications for breast cancer screening, prevention and therapy.

10. Conclusions

Of the more than 100,000 genes contained in the genome of the human breast cancer cell, only a few have been proven to be altered in malignant progression. HER-2/neu, c-*myc* and cyclin D1 are among oncogenes overexpressed and likely involved in the pathogenesis of human breast cancer. With information from studies of clinical cancer specimens, some distinct patterns of gene alteration are beginning to emerge. The results of several investigations suggest that the pathway to cancerous growth will likely involve cooperative interactions and networking connections among oncogenes, tumor suppressor and repair-mutator genes (Figure 5). The products of oncogenes and their cross-communication with growth factor and hormone signaling pathways also appear to play a major role in breast cancer progression. The challenge for the future is to identify the specific sequence and pattern of gene activation in breast cancer and to intensify our search for other crucial molecular defects leading to unregulated cell growth. Further, we must clarify the role of heritable gene alterations in this process. Hopefully, advances in this work will help us to devise novel therapeutics based on the unique biology of these cancers.

REFERENCES

1. Levine, A.J. (1995) The genetic origins of neoplasia. *J. Am. Medical Assoc.* **273**:592-593.

2. Li, F.P. and Fraumeni, Jr., J.F. (1969) Soft tissue sarcomas, breast cancer and other neoplasms: a familial syndrome? *Ann. Intern. Med.* **71**: 747-752.

3. Dickson, R.B. and Lippman, M.E. (1997) Cancer of the breast. In: *Cancer: Principles & Practice of Oncology*, 5th ed.(edited by DeVita, Jr., V.T., Hellman, S. and Rosenberg, S.A.), Lippincott-Raven Publishers, Philadelphia, pp. 1541-1557.

4. Miki, Y., Swansen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**: 66-71.

5. Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J. et al. (1995). Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**:789-792.

6. Hoskins, K. and Weber, B.L. (1995) Recent advances in breast cancer biology. *Curr. Opin. Oncol.* **7**:495-500.

7. Kinzler, K. and Vogelstein, B. (1997) Gatekeepers and caretakers. *Nature* **386**: 761-763.

8. Slamon, D., Press, M., Godolphin, W., Ramos, L., Haran, P., Shek, L., Stuart, S. and Ullrich, A. (1989). Studies of the HER-2/neu oncogene in human breast cancer. *Cancer Cells* **7** : 371-378.

9. Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J.M. and Meltzer, P.S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277**: 965-968.

10. Reese, D.M. and Slamon, D.J. (1997) HER-2/neu signal transduction in human breast and ovarian cancer. *Stem Cells* **15**:1-8.

11. Hynes, N.E. and Stern, D.F. (1994) The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim. Biophys. Acta* **1198**: 165-184.

12. Shih, C., Padhy, L., Murray, M., and Weinberg, R. (1981) Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* **260**: 261-264.

13. Bargmann, C., Hung, M. and Weinberg, R. (1986) Multiple independent activations of neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* **45**: 649-657.

14. Slamon, D.J., Clark, G.M., Wong, S.G. et al. (1987) Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**:177-182.

15. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J., Wong, S., Keith, D., Levin, W., Stuart, S., Udove, J., Ullrich, A., and Press, M.F. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707-712.

16. Pauletti, G., Dandekar, S., Smith, K., Robbins, P., Dawkins, H., Papadimitriou, J., Redmond, S., Harvey, J., Sterrett, G. and Slamon, D.J. (1997). Chronology of HER-2/neu gene amplification in proliferative and malignant ductal lesions of the breast as determined by fluorescence in situ hybridization (FISH). *Proc. Am. Assoc. Cancer Res.* **38**:414-415.

17. Liu, E., Thor, A., He, M., Barcos, M., Ljung, B.-M. and Benz, C. (1992) The HER2 (c-erbB-2) oncogene is frequently amplified in in situ carcinomas of the breast. *Oncogene* **7**, 1027-1032.

18. Pauletti, G., Godolphin, W., Press, M.F. and Slamon, D.J. (1996) Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* **13**, 63-72.

19. Lonn, U., Lonn, S., Nylen, U., Stenkvist, B. and Vennstrom, B. (1992) Detection and temporal appearance of multiple copies of c-erb-B2 genes in advanced mammary carcinoma using fine needle biopsies and the polymerase chain reaction. *Breast Cancer Research and Treatment* **23**: 191-200.

20. Iglehart, J.D., Kerns, B.-J., Huper, G., and Marks, J.R. (1995) Maintenance of DNA content and erbB-2 alterations in intraductal and invasive phases of mammary cancer. *Breast Cancer Research and Treatment* **34**, 253-263.

21. Press, M.F., Bernstein, L., Thomas, P.A., Meisner, L.F., Zhou, J.-Y., Ma, Y., Hung, G., Robinson, R.A., Harris, C., El-Naggar, A., Slamon, D.J., Phillips, R.N., Ross, J.S., Wolman, S.R. and Flom, K.J. (1997). HER-2/neu gene amplification characterized by fluorescence in situ hybridization: Poor prognosis in node-negative breast carcinomas. *J Clin Oncol.* **15**: 2894-2904.

22. Muss, H.B., Thor, A.D., Berry, D., Kute, T., Liu, E.T., Koerner, F., Cirrincione, C.T., Budman, D.R., Wood, W.C., Barcos, M. et al. (1994) c-erbB2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *New Engl. J. Med.* **330**: 1260-1266.

23. Berns, E.M.J.J., Foekens, J.A., van Staveren, I.L., van Putten W.L.J., de Koning, H.Y.W., Portengen, H. and Klijn, J.G.M. (1995) Oncogene amplification and prognosis in breast cancer: relationship with systemic treatment. *Gene* **159**, 11-18.

24. Fehm, T., Maimonis, P., Weitz, S., Teramoto, Y., Katalinic, A. and Jager, W. (1997) Influence of circulating c-erbB-2 serum protein on response to adjuvant chemotherapy in node-positive breast cancer patients. *Breast Cancer Research and Treatment* **43**: 87-95.

25. Nicholson, S., Wright,C., Sainsbury, J., Halcrow, P., Kelly, P., Angus, B., Farndon, J. and Harris, A.L. (1990). Epidermal growth factor receptor as a marker for poor prognosis in node-negative breast cancer patients : neu and tamoxifen failure. *J. Steroid Biochem.*. **37** : 811-818.

26. Wright, C., Nicholson, S., Angus, B., Sainsbury, J.R., Farndon, J., Cairns, J., Harris, A. and Horne, C. (1992) Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Brit. J. Cancer* **65** :118-124.

27. Benz, C., Scott, G., Sarup, J., Johnson, R., Tripathy, D., Coronado, E., Shepard, H. and Osborne, C. (1993) Estrogen-dependent, tamoxifen-resistent tumorigenic growth of MCF-7 cells transfected with HER2/neu. *Breast Cancer Res. Treatment* **24** :85-92.

28. Leitzel, K., Teramoto, Y., Konrad, K., Chinchilli, V., Volas, G., Grossberg, H., Harvey, H., Demers, L. and Lipton, A. (1995) Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer. *J. Clin. Oncol.* **13**: 1129-1135.

29. Pietras, R.J., Arboleda, J., Reese, D.M., Wongvipat, N., Pegram, M.D., Ramos, L., Gorman, C.M., Parker, M.G., Sliwkowski, M.X. and Slamon, D.J. (1995) HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* **10**: 2435-2446.

30. Pegram, M.D., Finn, R.S., Arzoo, K., Beryt, M., Pietras, R. and Slamon, D. (1997) The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. *Oncogene* **15**: 537-547.

31. Borg, A., Baldetorp, B., Ferno, M. et al. (1994) erb B2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. *Cancer Lett.* **81**: 137-144.

32. Pietras, R.J., Fendly, B.M., Chazin, V.R., Pegram, M.D., Howell, S.B. and Slamon, D. J. (1994) Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene* **9**: 1829-1838.

33. Arteaga C., Winnier, A., Poirier, M., Lopez-Larraza, D., Shawver, L., Hurd, S. and Stewart, S. (1994) p185c-erbB-2 signaling enhances cisplatin-induced cytotoxicity in human breast carcinoma cells: association between an oncogenic receptor tyrosine kinase and drug-induced DNA repair. *Cancer Res.*, **54**: 3758-3765.

34. Pegram, M., Lipton, A., Pietras, R., Hayes, D., Weber, B., Baselga, B., Tripathy, D., Twadell, T., Glaspy, J., and Slamon, D. (1995). Phase II study of intravenous recombinant humanized anti-p185 HER-2 monoclonal antibody (rhuMAb HER-2) plus cisplatin in patients with HER-2/neu overexpressing metastatic breast cancer. *Proc.Am.Soc.Clin.Oncol.* **14** : 106.

35. Carpenter, G.and Wahl, M.I. (1990) The epidermal growth factor family. *Peptide Growth Factors & Their Receptors* **1**: 69-171.

36. Carraway, K.L. III and Cantley, L.C. (1994) A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**: 5-8.

37. Soltoff, S.P., Carraway, K.L. III, Prigent, S.A., Gullick,W.G. and Cantley, L.C.(1994) ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol Cell Biol* **14**: 3550-3558.

38. Pawson,T. and Gish, G.D.(1992) SH2 and SH3 domains: from structure to function. *Cell* **71**: 359-362.

39. Batzer, A., Rotin, D., Skolnik, E.Y. and Schlessinger, J.F. (1994) Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol Cell Biol* **14**: 5192-5201.

40. Bollag, G. and McCormick, G. (1991) Regulators and effectors of ras proteins. *Annu. Rev. Cell Biol* . **7**: 601-632.

41. Chardin, P., Camonis, J.H., Gale, N.W., Van Aelst, L., Schlessinger, J., Wigler, M.H. and Bar-Sagi, D. (1993) Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. *Science* **260**: 1338-1346.

42. Earp, H.S., Dawson, T.L., Li, X. and Yu, H. (1995) Heterodimerization and functional interaction between EGF receptor family members: A new signaling paradigm with implications for breast cancer research. *Breast Cancer Res and Treat* **35**: 115-132.

43. Klijn, J.G.M., Berns, P.M.J.J., Schmitz, P.I.M. and Foekens, J.A. (1992) The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev* **13**: 3-17.

44. Normanno, N., Ciardiello, F., Brandt, R. and Salomon, D.S. (1994) Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. *Breast Cancer Res and Treat* **29**: 11-27.

45.Sainsbury, J., Farndon, J., Needham, G., Malcolm A. and Harris A.(1987) Epidermal growth factor receptor status as predictor of early recurrence of relapse and death from breast cancer. *Lancet* **I**:1398-1402.

46. Klijn, J., Berns, P., Schmitz, P. and Foekens, J. (1993) Epidermal growth factor receptor (EGFR) in clinical breast cancer: Update 1993. *Endocr Rev Monographs* **1**: 171-174.

47. Klijn, J., Look, M., Portengen, H., Alexieva-Figusch, J., van Putten, W. and Foekens, J. (1994) The prognostic value of epidermal growth factor receptor (EGFR) in primary breast cancer: Results of a 10 year follow-up study. *Breast Cancer Res and Treat* **29**: 73-83.

48. Nicholson, R., McClelland, R., Gee, J., Manning, D., Cannon, P., Robertson, J., Ellis, I. and Blamey R. (1994) Epidermal growth factor receptor expression in breast cancer: Association with response to endocrine therapy. *Breast Cancer Res and Treat* **29**: 117-125.

49. Van Agthoven, T., Van Agthoven, T., Portengen, H., Foekens, J., Dorssers, L. (1992) Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* **52**: 5082-5088.

50. Long, B., McKibben, B., Lynch, M. and Van den Berg, H. (1992) Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75-1 human breast cancer cell line. *Br J Cancer* **65**: 865-869.

51. Dickstein, B.M., Wosikowski, K. and Bates, S. (1995) Increased resistance to cytotoxic agents in ZR75B human breast cancer cells transfected with epidermal growth factor receptor. *Molec. and Cell Endocrinol.* **110**: 205-211.

52. Nass, S. and Dickson, R. (1997) Defining a role for c-myc in breast tumorigenesis. *Breast Cancer Res and Treat* **44**: 1-22.

53. Dubik, D., Dembinski, T.and Shiu, R.(1987) Stimulation of c-myc oncogene expression associated with estrogen-induced proliferation of human breast cancer cells. *Cancer Res.* **47**: 6517-6521.

54. Davidson, N., Prestigiacomo, L. and Hahm, H. (1993) Induction of jun gene family members by transforming growth factor a but not 17ß-estradiol in human breast cancer cells. *Cancer Res.* **53**: 291-297.

55. Leygue, E., Gol-Winkler, R., Gompel, A., Louis-Sylvestre, C., Soquet, L., Staub, S., Kuttenn, F., Mauvais-Jarvis, P. (1995) Estradiol stimulates c-myc proto-oncogene expression in normal human breast epithelial cells in culture. *J Steroid Biochen Molec Biol* **52**: 299-305.

56. Wenlu, L., Park, J.W., Nuijens, A., Sliwkowski, M.X. and Gilbert, A. (1996) Heregulin is rapidly translocated to the nucleus and its transport is correlated with c-myc induction in breast cancer cells. *Oncogene* **10**: 2473-2477.

57. Dubik, D. and Shiu, R. (1992) Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* **7**: 1587-1594.

58. Shiu, R., Watson, P. and Dubik, D. (1993) C-myc oncogene expression in estrogen-dependent and -independent breast cancer. *Clin. Chem.* **39**: 353-355.

59. Watson, P., Pon, R. and Shiu R. (1991) Inhibition of c-myc expression by phosphorothioate antisense oligonucleotide identifies a critical role for c-myc in the growth of human breast cancer. *Cancer Res.* **51**: 3996-4000.

60. Fernandez-Pol, J., Talkad, V., Klos, D. and Hamilton P. (1987) Suppression of the EGF-dependent induction of c-myc proto-oncogene expression by transforming growth beta in human breast carcinoma cell line. *Biochem. Biophys. Res. Commun* . **144**: 1197-1205.

61. Spence, M., Vestal, R. and Liu, J (1997) Oncostatin M-mediated transcriptional suppression of the c-myc gene in breast cancer cells. *Cancer Res.* **57**: 2223-2228.

62. Valverius, E., Ciardiello, F., Heldin, N., Blondel, B., Merlino, G., Smith, G., Stampfer, M., Lippman, M., Dickson, R. and Salomon, D. (1990) Stromal influences on transformation of human mammary epithelial cells overexpressing c-myc and SV40T. *J Cell Physiol* **145**: 207-216.

63. Schoenenberger, C., Andres, A., Groner, B., van der Valk, M., Lemeur, M., Gerlinger, P. (1988) Targeted c-myc gene expression in mammary glands of trangenic mice induces mammary tumors with constitutive milk protein gene transcription. *EMBO J* . **7**: 169-175.

64. Stewart, T., Pattengale, P. and Leder, P. (1984) Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell* **38**: 627-637.

65. Leder, A., Pattengale, P., Kuo, A., Stewart, T. and Leder, P. (1986) Consequences of widespread deregulation of the c-myc gene in trangenic mice: Multiple neoplasms and normal development. *Cell* **45**: 485-495.

66. Amundadottir, L., Johnson, M., Merlino, G., Smith, G. and Dickson, R. (1995) Synergistic interaction of transforming growth factor a and c-myc in mouse mammary and salivary gland tumorigensis. *Cell Growth Diff.* **6**: 737-748.

67. Andres, A., van der Valk, M., Schonenberger, C., Fluckiger, F., LeMeur, M., Gerlinger, P. and Groner, B. (1988) Ha-ras and c-myc oncogene expression interferes with morphological

and functional differentiation of mammary epithelial cells in single and double transgenic mice. *Genes Dev.* **2**: 1486-1495.

68. Sinn, E., Muller,W., Pattengale, P., Tepler, I., Wallace, R. and Leder, P. (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* **49**: 465-475.
69. Watson, P.H., Singh, R. and Hole, A. (1996) Influence of c-myc on the progression of human breast cancer. *Current Topics in Microbiology and Immunology* **213**:267-83.
70. Kozbor, D. and Croce, C.M. (1984) Amplification of the c-myc oncogene in one of five human breast carcinoma lines. *Cancer Res* . **44**: 438-441.
71. Bonilla, M., Ramirez, M., Lopez-Cueto, J. and Gariglio, P. (1988) In vivo amplification and rearrangement of c-myc oncogene in human breast tumors.*J. Natl. Cancer Inst* . **80**: 665-671.
72. Borg, A., Baldetrop, B., Ferno, M., Olsson, H. and Sigurdsson, H. (1992) c-myc is an independent prognostic factor in postmenopausal breast cancer. *Int .J. Cancer* **51**: 687-691.
73. Bieche,I.,Champeme,M.and Lidereau, R.(1994) A tumor suppressor gene on chromosome 1p controls the amplification of myc family genes in breast cancer. *Cancer Res.* **54**: 4274-4276.
74. Mizukami,Y., Nonomura, A., Takizawa, T., Noguchi, M., Michigishi, T., Nakamura, S. and Ishizaki, T. (1995) N-myc protein expression in human breast carcinoma: prognostic implications. *Anticancer Res* . **15**: 2899-2906.
75. Zhang, F. and Casey, P.J. (1996) Protein prenylation: molecular mechanisms and functional consequences. *Annual Review of Biochemistry* **65**:241-69.
76. Tocque, B., Delumeau, I., Parker, F., Maurier, F., Multon, M. and Schweighoffer, F. (1997) Ras-GTPase activating protein (GAP): a putative effector for Ras. *Cell.Signalling* **9**:153-158.
77. Clark, G. and Der, C. (1995) Aberrant function of the Ras signal transduction pathway in human breast cancer. *Breast Cancer Research and Treatment* **35**:133-44.
78. Spandidos, D. (1987) Oncogene activation in malignant transfromation: a study of H-ras in human breast cancer. *Anticancer Res.* **7**: 991-996.

79. Clair, T., Miller, W. and Cho-Chung, Y. (1987) Prognostic significance of the expression of a ras protein with molecular weight of 21,000 in human breast cancer. *Cancer Res* . **47**: 5290-5293.

80. Albini, A., Graf, J., Kitten, G., Kleinman, H., Martin, G., Veillette, A. and Lippman, M. (1986) 17ß-estradiol regulates and v-Ha-ras transfection constitutively enhances MCF-7 breast cancer cell interactions with basement membrane. *Proc.Natl.Acad.Sci. USA* **83**: 8182-8186.

81. Basolo, F., Elliott, J., Tait, L., Chen, X.Q., Maloney, T., Russo, I., Pauley, R., Momiki,S., Caamano, J., Klein-Szanto, A., Koszaika, M. and Russo, J. (1991) Transformation of human breast epithelial cells by c-Ha-ras oncogene. *Mol.Carcinogen* . **4**: 25-35.

82. Conway, K., Edmiston, S., Fried, D.B., Hulka, B.S., Garrett, P.A. and Liu, E.T. (1995) Ha-ras rare alleles in breast cancer susceptibility. *Breast Cancer Res.Treat* . **35**: 97-104.

83. Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E. and Auricchio, F. (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *EMBO J.* **15**:1292-300.

84. Archer, S., Eliopoulos, A., Spandidos, D., Barnes, D., Ellis, I., Blamey, R., Nicholson, R. and Robertson J. (1995) Expression of ras p21, p53 and c-erbB-2 in advanced breast cancer and response to first line hormonal therapy. *Oncogene* **72**: 1259-1266.

85. Kohl, N., Mosser, S., deSolms, S., Giuliani, E., Ponpliano, D., Graham, S., Smith, R., Scolnick, E., Oliff, A. and Gibbs, J. (1993) Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. *Science* **260**: 1934-1937.

86. Withers, D., Harvey, R., Faust, J., Melnik, O., Carey, K. and Meeker, T. (1991) Characterization of a candidate bcl-1 gene. *Mol.Cell.Biol* . **11**: 4846-4853.

87. Rosenberg, C., Kim, H., Shows, T., Kronenberg, H. and Arnold, A. (1991) Rearrangement and overexpression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors. *Oncogene* **6**: 449-453.

88. Schuuring, E. (1995) Involvement of the chromosome 11q13 region in human malignancy: cyclin D1 and EMS1 are two new candidate oncogenes - a review. *Gene* **159**: 83-96.

89. Sininski, P., Liu-Donaher, J., Parker, S.B., Li, T., Fazeli, A., Gardner, H., Haslam, S.Z., Bronson, R.T., Elledge, S.J. and Weinberg, R.A. (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**: 621-630.

90. Wang, T., Cardiff, R., Zukerberg, L., Lees, E., Arnold, A. and Schmidt, E. (1994) Mammry hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* **369**: 669-671.

91. Lovec, H., Grzeschiczek, A., Kowalski, M. and Moroy, T. (1994) Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. *EMBO J.* **13**: 3487-3495.

92. Quelle, D., Ashmun, R., Shurtleff, S., Kato, J., Bar-Sagi, D., Roussel, M. and Sherr, C. (1993) Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev.* **7**: 1559-1571.

93. Dickson, C., Fantl, V., Gillett, C., Brookes, S., Bartek, J., Smith, R., Fisher, C., Barnes, D. and Peters, G. (1995) Amplification of chromosome band 11q13 and a role for cyclin D1 in human breast cancer. *Cancer Lett* . **90**: 43-50.

94. van Diest, P., Michalides, R., Jannink, I., van der Valk, P., Peterse, H., de Jong, J., Meijer, C. and Baak, J. (1997) Cyclin D1 expression in invasive breast cancer: correlations and prognostic value. *Am. J. Pathol.* **150**: 705-711.

95. Michalides, R., Hageman, P., van Tinteren, H., Houben, L., Wientjens, E., Klompmaker, R. and Peterse, J. (1996) A clinicopathological study on overexpression of cyclin D1 and of p53 in a series of 248 patients with operable breast cancer. *Br. J. Cancer* **73**: 728-734.

96. Courjal, F., Louason, G., Speiser, P., Kataros, D., Zeillinger, R. and Theillet, C. (1996) Cyclin gene amplification and overexpression in breast and ovarian cancers: Evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int. J. Cancer* **69**: 247-253.

97. Porter, P., Malone, K., Heagerty, P., Alexander, G., Gatti, L., Firpo, E., Daling, J. and Roberts, J. (1997) Expression of cell-cycle regulators $p27^{Kip1}$ and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nature Medicine* **3**: 222-225.

98. Nielsen, N., Arnerlov, C., Emdin, S. and Landberg, G. (1996) Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. *Brit. J. Cancer* **74**: 874-880.

99. Rochefort, H. (1993) Response to P.M. Ravdin (BCRT 24: 219-226, 1993), "Evaluation of cathepsin D as a prognostic factor in breast cancer". *Breast Cancer Res.Trmt* . **26**: 107-111.

100. Himelstein, B., Canete-Soler, R., Bernhard, E. (1994) Metalloproteinases in tumor progression: The contribution of MMP-9. *Invasion Metastasis* **14**: 246-258.

101. Pietras, R.J., Szego, C.M., Roberts, J.A. and Seeler, B. (1981) Lysosomal cathepsin B-like activity: Mobilization in prereplicative and neoplastic epithelial cells. *J. Histochem. Cytochem.* **29**: 440-450.

102. Augereau, P., Miralles, F., Cavalles V. (1994) Characterization of the proximal estrogen-responsive element of human cathepsin-D gene. *Mol. Endocrinol.* **8**: 693-703.

103. Rochefort, H., Augereau, P., Briozzo, P. (1988) Structure, function, regulation and clinical significance of 52k procathepsin D secreted by breast cancer cells. *Biochimie* **70**: 943-949.

104. Vignon, F., Capony, F.and Chambon, M. (1986) Autocrine growth stimulation of the MCF-7 breast cancer cells by estrogen-regulated 52K protein. *Endocrinology* **118**: 1537-1545.

105. Pelizzola, D., Gion, M., Paradiso, A., Dittadi, R., Correale, M., Mione, R. and Piffanelli, A. (1996) Cathepsin D versus other prognostic factors in breast cancer. Results and controversies of a multicenter study on 2575 cases. *Int.J. Biological Markers* **11**: 139-147.

106. Nadji, M., Fresno, M., Nassiri, M., Gregory, C., Herrero, A. and Morales, A. (1996) Cathepsin D in host stromal cells, but not in tumor cells, is associated with aggressive behavior in node-negative breast cancer. *Human Pathol.* **27**: 890-895.

107. Ravdin, P:, de Moor, C., Hilsenbeck, S., Samoszuk, M., Vendely, P. and Clark, G. (1997) Lack of prognostic value of cathepsin D levels for predicting short term outcomes of breast cancer patients. *Cancer Letters* **116**: 177-183.

108. Gion, M., Mione, R., Dittadi, R., Romanelli, M., Pappagallo, L., Capitanio, G., Freide, U., Barbazza, R., Visona, A. and Dante, S. (1995) Relationship between cathepsin D and

other pathological and biological parameters in 1762 patients with primary breast cancer. *Eur. J. Cancer* **31A**: 671-677.

109. Pietras, R. and Szego, C. (1990) Cathepsin D in breast cancer. *New Engl. J. Med.* **322**:1673-1674.

110. Thomas, T. and Thomas, T. (1993) Extradiol control of ornithine decarboxylase mRNA, enzyme activity, and polyamine levels in MCF-7 breast cancer cells: therapeutic implications. *Breast Cancer Res.Treat.* **29**: 189-210.

111. Pegg, A., Secrist, J.A. III and Madhubala, R. (1988) Properties of L1210 cells resistant to alpha-difluoromethylornithine. *Cancer Res.* **48**: 2678-2682.

112. Martin, R., Ilett, K. and Minchin, R. (1991) Cell cycle-dependent uptake of putrexcine and its importance in regulating cell cycle phase transition in culture adult mouse hepatocytes. *Hepatology* **14**: 1243-1250.

113. Manni, A., Wechter, R., Grove, R., Wei, L., Martel, J.and Demers, L. (1995) Polyamine profiles and growth properties of ornithine decarboxylase overexpressing MCF-7 breast cancer cells in culture. *Breast Cancer Res.Treat.* **34**: 45-53.

114. Huber, M.and Poulin, R. (1996) Post-translational cooperativity of ornithine decarboxylase induction by estrogens and peptide growth factors in human breast cancer cells. *Molec. Cell. Endocrinol.* **117**: 211-218.

115. Manni, A. and Wright, C. (1984) Reversal of the antiproliferative effect of the antiestrogen tamoxifen by polyamines in breast cancer cells. *Endocrinology* **114**: 836-839.

116. Cohen, F., Manni, A., Glickman, P., Bartholomew, M. and Demers, L. (1988) Involvement of the polyamine pathway in antiestrogen-induced growth inhibition of human breast cancer. *Cancer Res.* **48**: 6819-6825.

117. Manni, A., Wechter, R., Wei, L., Heitjan, D.and Demers, L. (1995) Phenotypic features of breast cancer cells overexpressing ornithine-decarboxylase. *J. Cell.Physiol.* **163**: 129-136.

118. Persson, L.and Rosengren, E. (1988) Increased formation of N^1-acetylspermidine in human breast cancer. *Cancer Lett.* **45**: 83-86.

119. Wagner, A.J., Meyers, C., Laimins, L.A.and Hay, N. (1993) c-myc induces the expression and activity of ornithine decarboxylase. *Cell Growth Differ.* **4**: 879-883.

120. Wrighton, C.and Busslinger, M. (1993) Direct transcriptional stimulation of the ornithine decarboxylase gene by fos in PC12 cells but not in fibroblasts.*Mol.Cell.Biol.***13**:4657-4669.

121. Moshier, J., Dosescu, J., Skunca, M. and Luk, G. (1993) Transformation of NIH/3T3 cells by ornithine decarboxylase overexpression. *Cancer Res.* **53**: 2618-2622.

122. Kingsnorth, A., Wallace, H., Bundred, N. and Dixon, J. (1984) Polyamines in breast cancer. *Brit.J.Surg.* **71**: 352-356.

123. Halmekyto, M., Hyttinen, J., Sinervirta, R., Utrianen, M., Myohanen, S., Volpio, H., Wahlfors, J., Syrjanen, S., Syrjanen, K., Alhonen, L.and Janne, J. (1991) Transgenic mice aberantly expressing human ornithine decarboxylase gene.*J.Biol.Chem.* **266**: 19746-19751.

124. Mesa-Tejada, R., Keydar, I., Ramanarayanan, M., Ohno, T., Fenoglio, C. and Spiegelman, S. (1978) Detection in human breast carcinomas of an antigen immunologically related to a group-specific antigen of mouse mammary tumor virus. *Proc. Natl. Acad. Sci. USA* **75**: 1529-1533.

125. Litvinov, S. and Golovkina, T. (1989) Expression of proteins immunologically related to murine mammary tumor virus (MMTV) core proteins in the cells of breast cancer continuous lines MCF-7, T47D, MDA-231 and cells from human mild. *Acta Virologica* **33**: 137-142.

126. Day, N.K., Witkin, S.S., Sarkar, N.H., Kinne, D., Jussawalla, D.J., Levin, A., Hsia, C., Geller, N. and Good, R. (1981) Antibodies reactive with murine mammary tumor virus in sera of patients with breast cancer: Geographic and family studies. *Proc.Natl.Acad.Sci.USA* **78**: 2483-2487.

127. Callahan, R., Drohan, W., Tronick, S. and Schlom, J. (1982) Detection and cloning of human DNA sequences relaed to the mouse mammary tumor virus genome. *Proc.Natl. Acad.Sci.USA* **79**: 5503-5507.

128. Axel, R., Schlom, J. and Spiegelman, S. (1972) Presence in human breast cancer of RNA homologous to mouse mammary tumor virus RNA. *Nature* **235**: 32-36.

129. de Ricqles, D., Olomucki, A., Gosselin, F. and Ridereau, R. (1993) Breast cancer and T-cell-mediated immunity to proteins of the mouse mammary tumour virus (MMTV). *Eur. Cytokine Netw.* **4**: 153-160.

130. Crepin, M., Lidereau, R., Chermann, J., Pouillart, P., Magdamenat, H. and Montagnier, L. (1984) Sequences realted to mouse mammry tumor virus genome in tumor cells and lymphocytes from patients with breast cancer. *Biochem.Biophys.Res.Commun.* **118**: 324-331.

131. Al-Sumidaie, A.M., Hart, C.A., Leinster, S.J. and Green, C.D. (1988) Particles with properties of retroviruses in monocytes from patients with breast cancer. *Lancet* **1**: 5-8.

132. Pogo, B.G.-T. and Holland, J.F. (1997) Possibilities of a viral etiology for human breast cancer: A review. *Biol.Trace Element Res.* **56**: 131-142.

133. Rettig, M., Ma, H., Vescio, R., Pold, M, Schiller, G., Belson, D., Savage, A., Nishikubo, C., Fraser, J. and Berenson, J. (1997) Kaposi's sarcoma-associated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients.*Science* **276** :1851-1854.

134. Allred, D.C., Clark, G.M., Molina, R., Tandon, A.K., Schnitt S.J., Gilchrist, K.W., Osborne, C.K., Tormey, D.C. and McGuire, W.L. (1992) Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. *Human Pathology* **23**, 974-979.

135. King, M.-C., Rowell, S. and Love, S.M. (1993) Inherited breast and ovarian cancer. *J. Am. Med. Assoc.* **269**: 1975-1980.

136. Bieche, I. and Lidereau, R. (1995) Genetic alterations in breast cancer. *Genes, Chrom. & Cancer* **14**: 227-251.

137. Ruppert, J.M., Wright, M., Rosenfeld, M., Grushcow, J., Bilbao, G., Curiel, D. T. and Strong, T.V. (1997). Gene therapy strategies for carcinoma of the breast. *Breast Cancer Research and Treatment* **44**: 93-114.

138. Collins, K., Jacks, T., and Pavletich, N.K. (1997) The cell cycle and cancer. *Proc. Natl. Acad. Sci. USA* **94**, 2776-2778.

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Figure Captions

Figure 1. Hypothetical scheme for malignant progression of human breast cancer. Some genes may be inherited in altered form (Δ p53), deleted (- p53) or amplified (+ HER-2) during the course of breast cancer development and progression. Amplification of HER-2/neu gene is not found in normal breast tissue or hyperplasia / dysplasia (non-malignant tissue) but is found in ductal in situ carcinoma and in invasive ductal carcinoma of the breast. In the early stages of tumorigenesis, cyclin D gene expression appears to be prevalent in non-comedo ductal carcinoma in situ (96), while overexpression of HER-2/neu gene tends to predominate in comedo-type ductal carcinoma in situ (16). In more advanced breast malignancies, co-amplification of *c-myc* and HER-2/neu genes appears to occur infrequently in most studies, suggesting that activation of these oncogenes may represent independent avenues in breast cancer development. See text and independent reviews (134-136) for additional details on other potential gene alterations in breast cancer progression. Modified from Allred et al. (134), King et al.(135) and Bieche and Lidereau (136).

Figure 2. Metaphase spread of chromosomal material from a breast cancer cell line that shows amplification of the HER-2/neu gene. Fluorescence in situ hybridization (FISH) method was used to evaluate SKBR3 cells that have overexpression of HER-2/neu gene at the upper limit of that usually observed in clinical specimens. Punctate fluorescence is due to labeled probe specific for HER-2/neu gene (16). Provided by Dr. G. Pauletti.

Figure 3. Simplified model for growth factor receptor regulation of the growth of human breast cancer cells. The natural secretory products of mammary cells are abundant sources of growth factors that may contribute to breast carcinogenesis (3). HER-2/neu receptor is a transmembrane tyrosine kinase that forms a heterodimer with HER-3 and other EGF receptor-related proteins for binding growth factors such as heregulin (8,10,11). The HER-2/neu receptor signaling pathway is modulated by adaptor proteins, the mitogen-activated protein kinase (MAPK) pathway and the ras signaling pathway to promote changes in nuclear transcription. Downstream elements such as phospholipase C gamma, PI-3-kinase, GTPase activating protein and adaptor proteins such as SHC are part of the HER-2 receptor signaling machinery but are not shown here (see Figure 5; 10). In contrast to HER-2/neu, the tyrosine kinase catalytic site of HER-3 has absent or reduced kinase activity (36) and has sites which may afford specificity for activation of PI3 kinase (37). Cross-communication between HER-2/neu signaling and estrogen receptor (ER) signaling also occurs in breast cancer (29).

Figure 4. Representative photomicrograph of breast tumor tissue after fluorescence in situ hybridization (FISH) using a labeled probe specific for HER-2/neu gene (16). Example shows amplification of HER-2/neu gene as observed in a primary breast cancer specimen. Provided by Dr. G. Pauletti.

Figure 5. Hypothetical scheme for interaction of a growth factor receptor pathway with that of other tumor suppressor and proto-oncogene products in the regulation of cell growth. The cell cycle is controlled by an ordered series of cyclins, cyclin-dependent kinases (CDKs) and their inhibitors, such as p21/WAF1, which is modulated, in turn, by p53 gene products (1,41,42, 138). Growth factor receptors (HER-1, HER-2, HER-3, HER-4) and their respective ligands (EGF ligands - HER-1; heregulin ligand family - HER-3, HER-4), ras signaling pathways (ras, REF, MEK, MAP kinase) and c-*myc* gene products are also postulated to influence these regulatory events. Functional domains of c-*myc* are indicated, including the transcriptional activation domain (TAD), non-specific DNA-binding domain (NDB), basic specific DNA-binding domain (b), helix-loop-helix and leucine zipper oligomerization domains (HLH-ZIP; 136). See text for details.

Table 1. Proto-oncogene abnormalities and clinical correlates in human breast cancer*

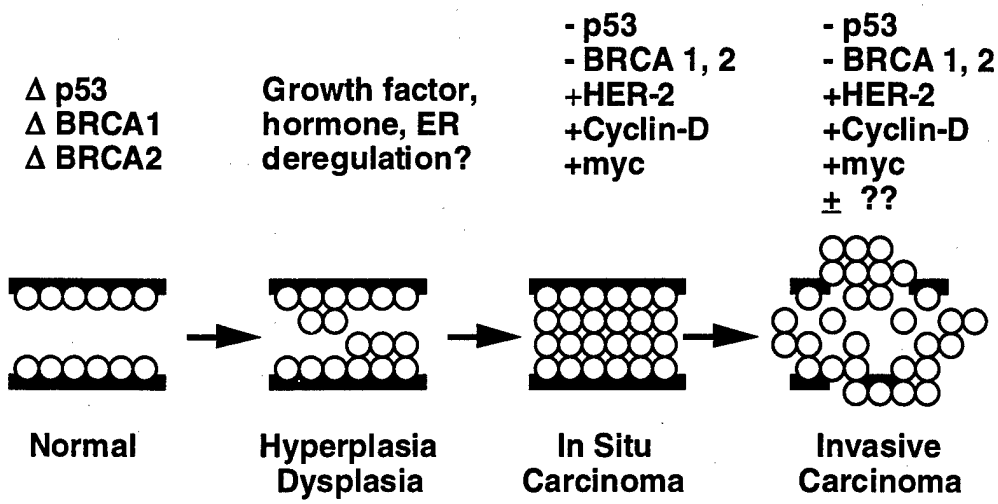
Proto-oncogene	Product	Abnormality	Clinical Correlate
HER-2/neu	185-kd membrane growth factor receptor	Gene amplification/ overexpression; Increased product	Poor prognosis; Poor response to therapy
HER-1/EGFR	170-kd membrane growth factor receptor	Gene overexpression	Poor prognosis?; Predicts response to therapy
c-myc	67-kd nuclear transcription factor	Gene amplification/ overexpression	Predicts early relapse, poor prognosis
c-ras	21-kd G-binding membrane protein	Amplification Point mutation Rearrangement	Does not correlate with overall survival
Cyclin D1/PRAD1	Regulator of G1-S transition	Gene overexpression	Correlates with estrogen receptor

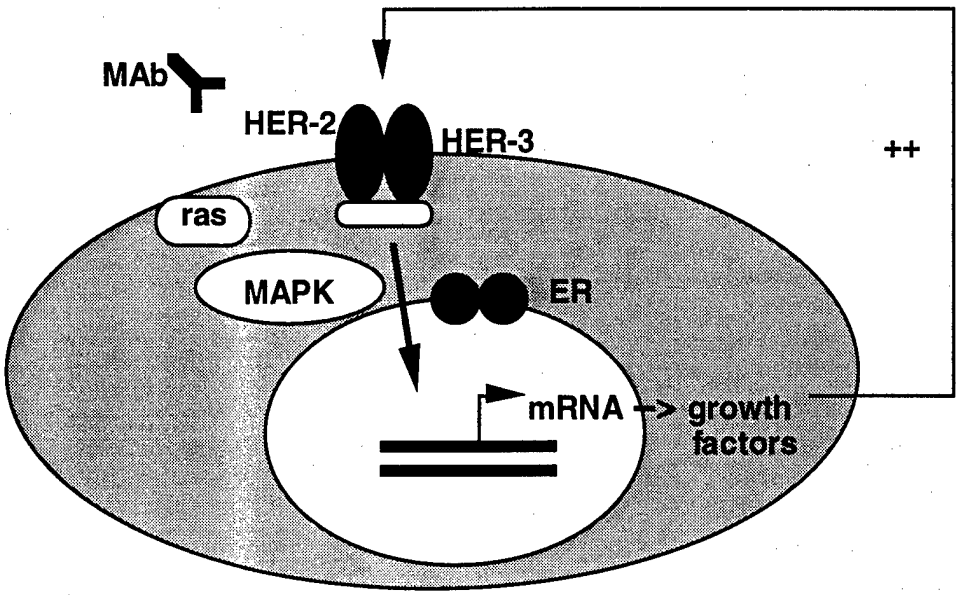
*Data derived from prior investigations on HER-2 (3,6,8, 14,15), EGFR (43-48), *c-myc* (52,69-74), c-ras (77-81) and cyclin D1 (93-96).

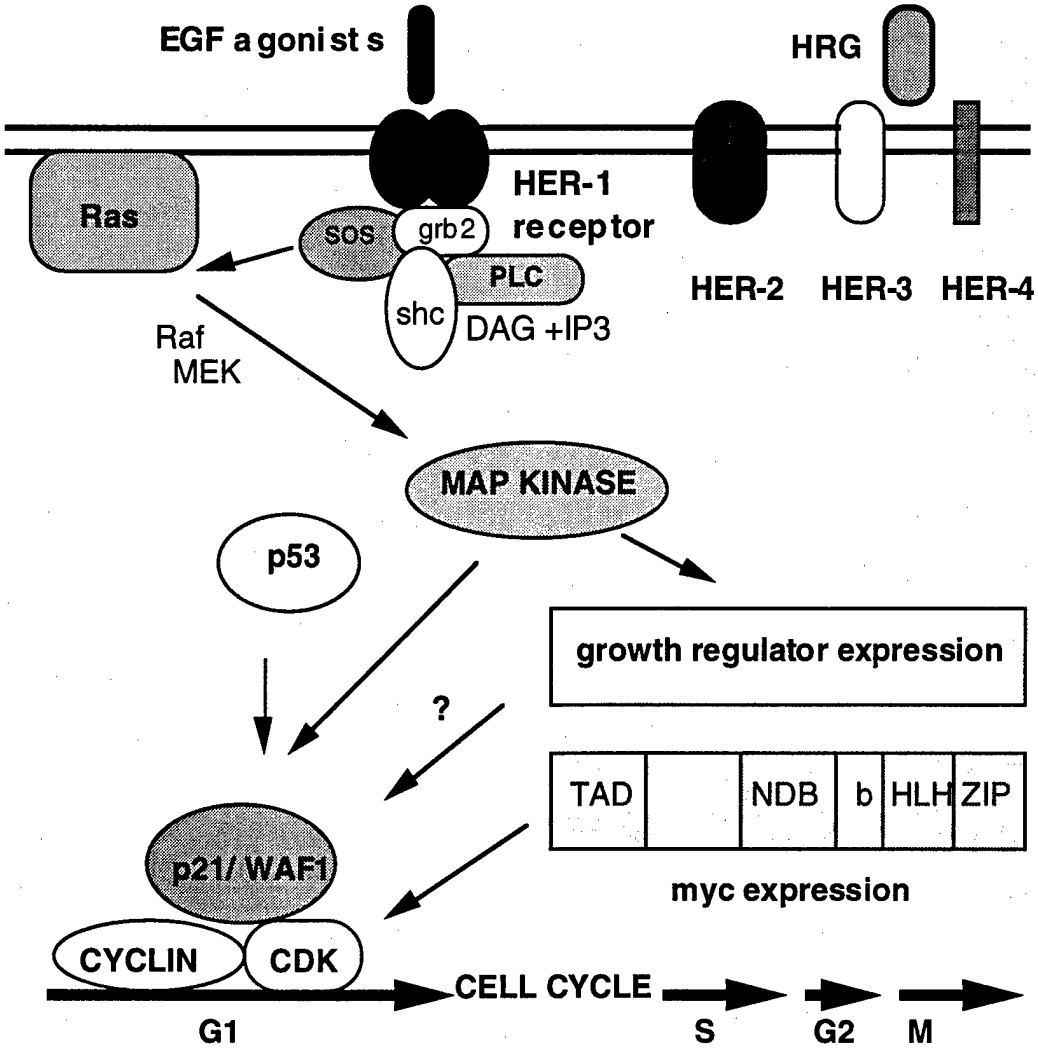
Table 2. Candidate proto-oncogenes and clinical correlates in human breast cancer*

Candidate Gene	Product	Abnormality	Clinical Correlate
AIB1	Steroid receptor co-activator	Gene amplification / overexpression	?
int-2	27-kd protein	Gene amplification	?
FLG, BEK	Fibroblast growth factor receptors	Gene amplification	?
IGFR	Insulin-like growth factor receptor	Gene amplification	?
Ornithine Decarboxylase	Enzyme in polyamine biosynthesis	Gene overexpression	?
Cathepsin D	Proteinase	?	Poor prognosis?
MMTV*env* -like gene	Undefined	?	?

*Data derived from prior investigations on AIB1 (9), int-2 (3, 136,137), FLG, BEK, IGFR (3, 136,137), ornithine decarboxylase (110,118), cathepsin D (105-108) and MMTV env-like genes (124-132).


Δ p53
Δ BRCA1
Δ BRCA2
Growth factor, hormone, ER deregulation?
- p53
- BRCA 1, 2
+HER-2
+Cyclin-D
+myc
- p53
- BRCA 1, 2
+HER-2
+Cyclin-D
+myc
± ??
Normal
Hyperplasia Dysplasia
In Situ Carcinoma
Invasive Carcinoma


MAb
HER-2
HER-3
++
ras
MAPK
ER
mRNA -> growth factors


EGF agonists
HRG
Ras
SOS
grb2
HER-1
receptor
PLC
shc
DAG +IP3
HER-2
HER-3
HER-4
Raf
MEK
MAP KINASE
p53
growth regulator expression
?
TAD
NDB
b
HLH
ZIP
myc expression
p21/ WAF1
CYCLIN
CDK
CELL CYCLE
G1
S
G2
M

In preparation

Reversal of tamoxifen resistance in HER-2/neu-overexpressing human breast cancer cells using HER-2/neu antibody

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Abbreviations: ER, estrogen receptor; ERE, estrogen-responsive elements; MCF-7/HER-2, MCF-7 breast cancer cells with overexpression of human HER-2/*neu* gene; CAT, chloramphenicol acetyltransferase.

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ABSTRACT Antiestrogen therapy has significantly improved overall survival in patients with breast cancer due to its ability to alter the regulation of breast cell growth by estrogens. Approximately 30% of breast cancer patients, however, overexpress the HER-2/*neu* growth factor receptor, and this abnormality is associated with estrogen-independent growth as well as a poor response to tamoxifen therapy. To evaluate this phenomenon, human breast cancer cells with and without HER-2/*neu* overexpression were grown as subcutaneous xenografts in nude mice. As expected, *in vivo* growth of estrogen-dependent, estrogen receptor-positive MCF-7 cells was completely suppressed by tamoxifen, a classical antiestrogen, as well as by ICI 182,780, a pure antiestrogen. In contrast, MCF-7 cells with HER-2/*neu* receptor overexpression were completely resistant to tamoxifen and only partially sensitive to ICI 182,780. Treatment of HER-2/*neu*-overexpressing MCF-7 cancer cells with a monoclonal antibody directed against HER-2/*neu* receptor in combination with tamoxifen resulted in restoration of tamoxifen sensitivity to levels found in MCF-7 cells without overexpression. Moreover, in cells containing HER-2/*neu* overexpression, estradiol further promotes interaction between estrogen receptor and estrogen-responsive elements. This latter effect is blocked by ICI 182,780 but not by tamoxifen. Treatment with tamoxifen combined with HER-2/*neu* antibody, however, reestablishes the growth inhibitory effects of tamoxifen. This therapeutic effect of antireceptor antibody in combination with tamoxifen may be related, in part, to the state of tyrosine phosphorylation of estrogen receptor. These data indicate that binding of HER-2/*neu* receptor by antireceptor antibody enhances the therapeutic efficacy of antiestrogens in human breast cancer cells which overexpress HER-2/*neu* oncogene.

In preparation

Biologic Effects of Heregulins on Normal and Malignant Human Breast and Ovarian Epithelial Cells

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Key Words: Heregulin, NDF, HER-2/neu, erb B2, growth factor, epithelial cells, membrane-anchored ligand

Final Report Bibliography

PUBLICATIONS

1) Pietras, R.J., B.M. Fendly, V. Chazin, M.D. Pegram, S.B. Howell and D.J. Slamon (1994). Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene 9 : 1829-1838.

2) Pietras, R. J., J. Arboleda, D. Reese, N. Wongvipat, M. Pegram, L. Ramos, C. M. Gorman, M.G. Parker, M. X. Sliwkowski, and D. J. Slamon (1995). HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene 10 : 2435-2446.

3) Pegram, M.D., R. Finn, K. Arzoo, M. Beryt, R. J. Pietras and D. J. Slamon (1997). The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene 15: 537-547.

4) Pietras, R.J. (1997). HER-2 tyrosine kinase pathway regulates estrogen receptor and growth in human breast cancer cells. Proceedings DOD Breast Cancer Research Program 3: 987-988.

5) Pegram, MD, S. Hsu, R. Pietras, M. Sliwkowski, M. Beryt, D. Coombs, D. Baly, F. Kabbinavar and D. Slamon (1998). Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene (in press).

6) Pietras, R.J. and M. Pegram (1998). Oncogene activation and breast cancer progression. Contemporary Endocrinology, 11 (in press).

7) Pietras, R.J., M. Pegram, R. Finn, D. Maneval and D. Slamon (1998). Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive agents. Oncogene (in press).

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8) Aguilar, Z., R. Finn, M. Pegram, B. Ramos, F. Kabbinavar, R.J. Pietras, R. Akita, M. Sliwkowski and D. Slamon (1999). Biologic effects of heregulins on normal and malignant human breast and ovarian epithelial cells. (To be submitted; in preparation).

9) Pietras, R.J., P.N. Wongvipat and D.J. Slamon (1999). Antibody to HER-2/neu growth factor receptor reverses antiestrogen resistance in human breast cancer cells with overexpression of HER-2/neu oncogene. (To be submitted; in preparation).

10) Pietras, R.J., P.N. Wongvipat, H.J. Lee and D.J. Slamon (1999). Monoclonal antibody to HER-2 receptor modulates WAF1/p21 activity and the repair of cisplatin-induced DNA damage in human breast cancer cells. (To be submitted; in preparation).

ABSTRACTS

1) Pietras, R.J., J. Arboleda, N.Wongvipat, L. Ramos, M.Sliwkowski, and D.J. Slamon (1995). HER-2/neu signaling regulates estrogen receptor in breast cancer. Proc. Am. Assoc. Cancer Res. 36 : 254.

2) Pegram, M., A. Lipton, R. Pietras, D. Hayes, B. Weber, J. Baselga, D. Tripathy, T. Twadell, J. Glaspy and D. Slamon (1995). Phase II study of intravenous recombinant humanized anti-p185 HER-2 monoclonal antibody (rhuMAb HER-2) plus cisplatin in patients with HER-2/neu overexpressing metastatic breast cancer. Proc. Am. Soc. Clin. Oncol. 14 : 106a.

3) Hsu, S., M. Pegram, R. Pietras, M. Beryt and D. Slamon (1997). Therapeutic advantage of chemotherapy drugs in combination with recombinant, humanized anti-HER-2/neu monoclonal antibody (rhuMAb HER-2) against human breast cancer cells and xenografts with HER-2/neu overexpression. Proceedings of AACR Conference on Basic and Clinical Aspects of Breast Cancer 3/12: A39

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